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The fatty acids of beef brain and spinal cord sphingolipid preparations*

K. K. Carroll

Collip Medical Research Laboratory,
University of Western Ontario,
London, Canada

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SUMMARY

Sphingolipid preparations from beef spinal cord and from a mixture of beef brain and beef spinal cord were subjected to methanolysis, and the composition of the fatty acid esters was investigated by conventional fractional distillation, by column chromatography, and by gas-liquid chromatography. Approximately 20 fatty acids were separated and characterized. Some improvements in methodology are reported in connection with these analyses.

Our interest in sphingolipid preparations arose from studies on the effects of fatty acids of chain length greater than C_{18} on cholesterol metabolism. When fed to rats, certain of these fatty acids (particularly erucic and nervonic acid) raised the level of cholesterol in some tissues and increased fecal excretion of cholesterol (1, 2). Since nervonic acid was a known constituent of cerebrosides and since feeding sphingolipid preparations from beef brain and spinal cord also increased fecal sterol excretion, further studies were carried out to determine if the effect of the sphingolipid preparations was related to the nature of their fatty acid components (3). As a corollary to this work, a quantitative study was made of the fatty acid composition of the spingolipid preparation from beef spinal cord, and the results are presented in this report. Data are also given on the fatty acid composition of commercial preparations of sphingolipids from beef spinal cord and from a mixture of beef brain and beef spinal cord.

EXPERIMENTAL

Most of the analytical work was done on crude beef spinal cord sphingolipids prepared in our laboratory by the method of Carter *et al.* (4). The yields and appearance of the sphingolipid fractions were essentially as reported by these workers, and the results of sugar and phosphorus analyses on our preparations (3) were compatible with the composition they suggested—namely, 71% cerebrosides, 23.5% sphingomyelin, and 5.5% of other material.

In our experiments, the connective tissue was not generally removed from the spinal cord prior to extraction, but in one instance 21.4 kg of frozen spinal cord was partially thawed and the connective tissue covering was completely stripped off. This left 15.5 kg of cord, which was extracted in the usual manner. The residues after extraction with acetone and ether were 4.6 and 2.9 kg, respectively, and the sphingolipid fraction that precipitated from the hot alcohol extract weighed 1.06 kg. This yield of sphingolipid is similar to that obtained by extracting the cords plus connective tissue and indicates that the connective tissue contributes relatively little to this fraction.

The sphingolipid preparations were subjected to methanolysis, and the methyl esters of the fatty acids were recovered by methods also described by Carter and his associates (5). The mixture of methyl esters obtained in this way and used in our animal experiments and in clinical studies on patients with multiple sclerosis (6) was referred to as FAF (fatty acid fraction) oil. More recently, beef spinal cord sphingolipids have been prepared by a modified extraction procedure in the laboratories of Canada Packers, Ltd., Toronto, Canada, for use in a larger-scale clinical trial; the mixture of methyl esters obtained from this preparation was called Hyfalean. Comparative studies were done on the fatty acid composition of this preparation. Some work was also done on a sphingolipid preparation (WT-5) from a mixture of beef brain and beef spinal

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TABLE 1. MAJOR COMPONENT FATTY ACIDS OF SPHINGOLIPID PREPARATIONS

Fatty Acid Ester	Shorthand Designation	Relative t_R* on SE-30	Relative t_R* on diethyleneglycol succinate	Percentage Composition Wilson	Hyfalean†	FAF oil
Myristic	(14:0)	0.27	0.28	0.6	0.3	0.4
	(15:0)	0.38	0.38	0.3	1.1	0.2
Palmitic	(16:0)	0.50	0.54	12.5	4.2	3.2
	(17:0)	0.73	0.73	0.8	1.2	0.6
Oleic	(18:1)	0.93	1.18	10.0	10.1	4.0
Stearic	(18:0)	1.00	1.00	19.7	12.5	11.5
	(19:0)	1.39		0.8	0.9	1.0
α-Hydroxystearic	(18:0) (OH)	1.51	4.46	8.0	7.6	7.3
Eicosenoic	(20:1)	1.77	2.09	4.8	7.7	4.5
Arachidic	(20:0)	1.96	1.80	1.3	2.0	2.9
Erucic	(22:1)	3.37	3.78	1.0	1.4	1.2
Behenic	(22:0)	3.75	3.31	3.1	4.2	7.5
Tricosanoic	(23:0)	5.17		1.7	2.0	2.9
α-Hydroxybehenic	(22:0) (OH)	5.70		1.1	1.8	1.6
Nervonic	(24:1)	6.50	7.03	17.5	12.9	17.3
Lignoceric	(24:0)	7.16	6.18	7.7	11.8	15.5
α-Hydroxytricosanoic	(23:0) (OH)	7.90		1.1	1.8	1.9
?		8.95		1.7	1.7	1.5
α-Hydroxynervonic	(24:1) (OH)	9.9		1.8	4.3	3.8
α-Hydroxylignoceric	(24:0) (OH)	10.8		3.1	8.2	8.0
?		12.4		1.2	2.0	3.0

* In cases where no retention time is given for diethyleneglycol succinate, the fatty acid was either not eluted from the polar column or its peak on SE-30 could not be definitely correlated with a peak on the polar column. In a few cases, the expected retention time on the polar column coincided with that of a more major component.

† Lot number 341-40.

cord prepared by the Wilson Co., Ltd., Chicago, Illinois, and made available to us through the courtesy of Dr. David Klein. The fatty acid esters were prepared in our laboratory as described above. The yields of methyl esters from our own preparation and from the Wilson preparation amounted to 45% to 50% of the total weight of the original material. The iodine values (Hanus) and saponification equivalents of the various preparations were, respectively: Wilson preparation, 39 and 327; Hyfalean, 37 and 366; FAF oil, 30 and 365.

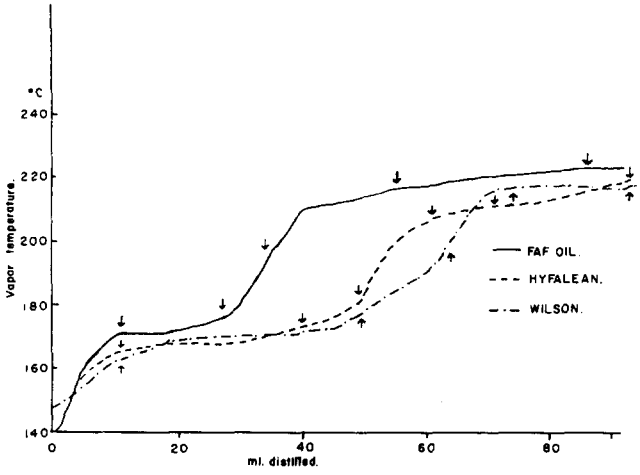

FIG. 1. Fractional distillation of methyl esters of the fatty acids from sphingolipid preparations. The distillates were separated into fractions with different boiling ranges at the points indicated by the arrows.

One-hundred-gram aliquots of the methyl esters from the various preparations were subjected to fractional distillation in a Todd still (Todd Scientific Co., Springfield, Pennsylvania). The complete mixtures and the fractions obtained from the distillations were also analyzed by gas-liquid chromatography using a Barber-Colman chromatograph. Most of the fractions were analyzed both on a nonpolar column (10% SE-30 on Chromosorb W) and on a polar column (20% diethyleneglycol succinate on Chromosorb W). Another polar column (20% butanediol succinate on Chromosorb W) was used in a few experiments. Diethyleneglycol succinate was obtained from the Cambridge Instruments Co., Ltd., Cambridge, Massachusetts; butanediol succinate and SE-20 from Wilkins Instrument and Research, Inc., Walnut Creek, California; and Chromosorb W, 60 to 80 mesh, from the Johns Manville Corp., Toronto, Canada.

TABLE 2. SEPARATION OF HYDROXYLATED AND NONHYDROXYLATED FATTY ACID ESTERS OF SPHINGOLIPID PREPARATIONS BY FLORISIL CHROMATOGRAPHY

Preparation	Amount Chromatographed	Nonhydroxylated Esters	Hydroxylated Esters	Total Recovery
	mg	%	%	%
Wilson	550	76	18	94
Hyfalean	550	59	29	88
FAF oil	500	63	26	89
Fraction 1	200	94	1	95
Fraction 2	200	78	24	102
Fraction 3	200	47	45	92
Fraction 4	200	76	12	88
Fraction 5	200	68	27	95

Hydroxylated fatty acids were separated from nonhydroxylated acids by chromatographing their methyl esters on 30-g columns of Florisil (7, 8). The nonhydroxylated esters were eluted with 150 ml of 5% ether in Skellysolve B (Skelly Oil Co., Kansas City, Missouri), and the hydroxylated esters were eluted with 250 ml of 25% ether in Skellysolve B. Florisil (activated magnesium silicate) was obtained from the Floridin Co., Tallahassee, Florida. Material activated either at 260° or 650° was used. The latter was deactivated before use by addition of 7% by weight of water.

RESULTS

Distillation curves obtained from fractional distillation in the Todd still of methyl esters of the fatty acids from three sphingolipid preparations are shown in Figure 1. Gas-liquid chromatograms obtained with methyl esters from FAF oil are shown in Figure 2. Chromatograms of methyl esters from the other sphingolipid preparations showed that they contained the same major component fatty acids although the proportions varied somewhat. Percentage compositions and retention times of the methyl esters of the fatty acids relative to methyl stearate are given in Table 1. The percentage compositions were derived from gas-liquid chromatographic analyses of the complete mixtures together with chromatographic analyses of the fractions obtained by conventional fractional distillation (Fig. 1) and by Florisil chromatography (Table 2). The product of peak height and retention time was used as a measure of peak area on the gas-liquid chromatograms (9), and correction factors were applied to allow for the nonlinear response of the detector.

Gas-liquid chromatographic analysis of the fractions obtained by fractional distillation (Fig. 1) showed that

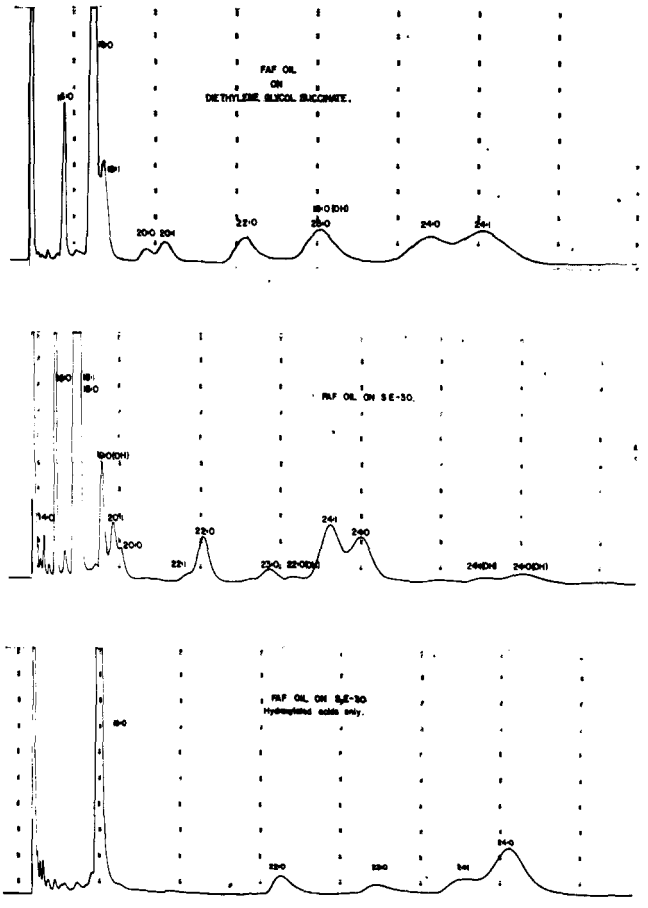

FIG. 2. Gas-liquid chromatographic separation of methyl esters of the fatty acids from a beef spinal-cord sphingolipid preparation (FAF oil) on a Barber-Colman chromatograph equipped with a radium detector. Glass columns (6′ × 1/4″) packed with either 10% SE-30 or 20% diethyleneglycol succinate on Chromosorb W (60 to 80 mesh) were used for the analyses. The SE-30 column was operated at 216° and 20 lb/sq in. of argon at the inlet. The diethyleneglycol succinate column was used at 192° and 14 lb/sq in. argon. The first peak at the left of each chart is due to solvent.

fractions 1 and 2 contained only C_{16} and C_{18} acids. Fraction 3 contained 50% to 60% of C_{20} acids together with hydroxystearic acid and a small amount of stearic acid. Fraction 4 of the Hyfalean consisted mainly of C_{22} acids with only small amounts of C_{20} and C_{24} acids. Fraction 4 of the Wilson preparation and of FAF oil contained 30% and 50% respectively of C_{24} acids. Fractions 5 and 6 consisted almost entirely of C_{24} or longer fatty acids. These data and the gas-liquid chromatographic analyses of the complete mixtures indicated that FAF oil contained the highest proportion of fatty acids with chain length greater than C_{18} and the Wilson preparation contained the least.

Identification of the component fatty acids was accomplished by a variety of procedures. The retention times of myristic, palmitic, oleic, stearic, α-

hydroxystearic, eicosenoic, arachidic, erucic, behenic, α-hydroxybehenic, nervonic, and lignoceric acids were compared with those of authentic known standards on both polar and nonpolar gas-liquid chromatographic columns. Standards were not available for odd-chain fatty acids, but the peaks designated as 15:0, 17:0, 19:0, and 23:0 had the expected retention times based on a semi-log plot of retention time against chain-length obtained with other saturated fatty acid standards. There were at least two other components with relatively long retention times that were present in rather small amounts and appeared to be saturated nonhydroxylated acids. Their retention times did not seem to fall on the semilog plot of retention time against chain length, but this could be due to inaccuracies in determining the retention times of long, low peaks that might not be due to single components.

Bromination and hydrogenation were used to distinguish between saturated and unsaturated fatty acids (10). The relative positions of the components during gas-liquid chromatography on polar and on nonpolar stationary phases indicated degree of unsaturation.

Further identification of the unsaturated C_{20} and C_{22} acids was obtained in the following way. Fractions in which each of these was the major unsaturated fatty acid were obtained by fractional distillation in the Todd still. These fractions were then oxidized to split double bonds, and the resulting mixtures were methylated and rechromatographed (11). Peaks corresponding to eicosenoic acid and erucic acid were eliminated by this procedure and new peaks appeared, corresponding to C_{11} and C_{13} dibasic acids, respectively. These results indicated that the double bond in the C_{20} acid was in the 11,12-position and the double bond in the C_{22} acid was in the 13,14-position. The retention times of the methyl esters of the dibasic acids were determined by oxidizing authentic samples of the monounsaturated acids. The retention times relative to methyl stearate on a 6-ft column packed with 10% SE-30 on Chromosorb W and operated at 200° were 0.11, 0.23, and 0.49 for the C_9, C_{11}, and C_{13} esters, respectively. Methyl stearate had a retention time of 11.2 minutes under these conditions.

The hydroxylated fatty acids were separated from the nonhydroxylated acids by chromatographing their methyl esters on Florisil columns. Results obtained with fatty acid esters from the different sphingolipid preparations and from the individual distilled fractions of FAF oil are shown in Table 2.

With the exception of α-hydroxystearic acid, the methyl esters of α-hydroxy acids had retention times on the polar phases that were too long for convenient analysis by gas-liquid chromatography. However, the methyl esters of the corresponding α-methoxy acids prepared by the method of Kishimoto and Radin (7) had much shorter retention times and could be analyzed satisfactorily. The presence of a hydroxyl group did not cause nearly as much retardation on the nonpolar SE-30 column, and methyl esters of hydroxy acids up to C_{24} could be analyzed directly on this type of column. Conversion of the hydroxy group to a methoxy group shortened the retention times only slightly on SE-30 (Table 3).

Hydroxystearic and hydroxylignoceric acids were the major hydroxylated fatty acids of the sphingolipid preparations (Fig. 2). Smaller amounts of hydroxybehenic, hydroxytricosanoic, and hydroxynervonic, and a trace of hydroxyarachidic were also present. Some minor peaks with short retention times were not identified. Hydroxystearic was the only hydroxylated fatty acid in fractions 2 and 3 of the FAF oil distilled in the Todd still (Table 2). Hydroxybehenic was the major hydroxylated acid in fraction 4, and hydroxylignoceric was the main one in fraction 5.

The saturated hydroxy acids were identified on the basis of their retention times on the different phases and their failure to be affected by hydrogenation. Hydroxynervonic acid was the only unsaturated hydroxy acid present in appreciable amounts in these preparations. The peak corresponding to hydroxynervonic acid was eluted after hydroxylignoceric acid on butanediol succinate[1] and before hydroxylignoceric on SE-30, and the positions relative to the saturated acid were those expected for a monounsaturated acid.

DISCUSSION

Crude sphingolipid preparations from brain and spinal cord contain a mixture of hydroxylated and nonhydroxylated fatty acids varying in chain length up to C_{24} and longer. Although gas-liquid chromatography has greatly simplified the analysis of such mixtures, exact quantitative analysis still presents problems. The ionization detector used in the present studies did not give strictly linear increases in peak area for increasing amounts of any given ester, and esters of chain length C_{20} and above gave less area per unit weight than those of chain length C_{16} and C_{18}. Similar deviations with an ionization detector have been reported by Tandy *et al.* (12). It was therefore undesirable with this detector[2] to make direct comparisons between peaks differing greatly in height or between

[1] The α-methoxy esters were chromatographed in this experiment.

[2] The new 1-cm ionization detector now available appears to give a much more linear response.

esters of very different chain length. Consequently, in our studies, gas-liquid chromatographic analysis was applied not only to the complete mixtures of fatty acids from the sphingolipid preparations but also to fractions obtained by conventional fractional distillation and by column chromatography in order to obtain the best possible analytical results. Some lack of agreement was observed between results obtained by direct analysis of the complete mixtures and the results obtained by analyzing the fractions and, from the composition and amounts of these, calculating the composition of the original mixture. The figures given, therefore, represent a compromise but it is felt that they give close approximations to the correct analyses.

The methods used in our work were in some respects an improvement on those used in previous analyses of fatty acids of myelin lipids (7, 13, 14, 15). The separation of hydroxylated and nonhydroxylated acids by column chromatography was facilitated by using less active Florisil and by increasing the proportion of ether in the eluting solvents. Thirty-gram columns were used in our experiments because rather large amounts of esters were fractionated in some cases. For fractionation of smaller amounts, the quantity of Florisil could presumably be reduced with consequent saving in the quantity of eluting solvents used (8).

TABLE 3. RETENTION TIMES OF METHYL ESTERS OF HYDROXY AND METHOXY ACIDS RELATIVE TO METHYL STEARATE ON POLAR AND NONPOLAR COATINGS

Ester	Relative Retention Time*		
	On Diethyleneglycol Succinate	On Butanediol Succinate	On SE-30
α-Hydroxystearic	4.5	3.6 (1.87)	1.51 (1.45)
α-Hydroxybehenic		(6.0)	5.7 (4.9)
α-Hydroxylignoceric		(10.7)	10.8 (8.9)

* Values for the corresponding methoxy derivatives are given in parentheses.

Previous gas-liquid chromatographic separations of the fatty acids of myelin lipids were generally done on polyester columns, which were not satisfactory for the analysis of hydroxylated acids of chain length greater than C_{18} unless the hydroxyl group was modified (7). Johnston *et al.* (15) used a 10-ft silicone rubber column and were able to elute α-hydroxy methyl esters up to C_{24} but found that the peaks were markedly skewed. Our experience with the nonpolar SE-30 column[3] indicated that it was suitable for analysis of α-hydroxy methyl esters up to C_{24}. The retention times of the major hydroxy esters were sufficiently different from those of the nonhydroxylated esters in sphingolipid preparations to permit a direct comparison of both types on the same chromatogram (Fig. 2). This was a considerable advantage for analytical work. The relative retention times of fatty acid esters on the SE-30 column seemed to be more sensitive to changes in the operating conditions than the relative retention times on polar columns, and the peaks were less symmetrical; with the availability of known acids for comparison, these were not serious problems.

It is well known that cerebrosides contain mainly lignoceric acid, nervonic acid, and their corresponding α-hydroxy acids, while sphingomyelins from various sources contain palmitic, stearic, lignoceric, and nervonic acids (16). More recently, α-hydroxystearic acid has been identified as a component of beef spinal-cord cerebrosides (17). Behenic acid has also been characterized as a component of cerebrosides (18) and of sphingomyelin (19). All of these fatty acids were present in our sphingolipid preparations from beef spinal cord.

Kishimoto and Radin (13) reported the presence of saturated and unsaturated fatty acids with and without an α-hydroxy group of all chain lengths from C_{20} to C_{24} in rat-brain cerebroside preparations. The presence of C_{25} and C_{26} acids was indicated by these authors and was also reported by Radin and Akahori (20) and by Johnston and Kummerow (14). Our preparations contained little, if any, of the C_{21} acids, and the only unsaturated hydroxy acid present in any quantity was hydroxynervonic acid. Most of the other acids observed by Kishimoto and Radin were present, and C_{24} acids predominated in the group with chain lengths greater than C_{18}. Evidence was obtained from our chromatograms of fatty acids longer than C_{24}; as in previous studies, however, characterization was difficult because of the small amounts present and the problem of determining exact retention times for long, low peaks.

Kishimoto and Radin (13) provided evidence that the hydroxylated fatty acids in rat-brain cerebrosides were α-hydroxy acids, and Skipski *et al.* (17) established the identity of α-hydroxystearic acid. It seemed likely, therefore, that the hydroxylated acids found in our preparations were also α-hydroxy acids. In this group, the C_{18} and C_{24} saturated acids were present in largest amounts.

The unsaturated C_{20} and C_{22} acids in our preparations were identified by oxidation of the double bond as Δ11-eicosenoic and Δ13-erucic acids, which could be biosynthesized by addition of 2-carbon fragments to oleic acid. The C_{24} unsaturated acid, nervonic acid, could be formed in a similar way since it has a double bond

[3] The use of SE-30 for the separation of fatty acid esters was suggested to the author by Dr. N. S. Radin.

in the Δ15-position. However, the presence of a Δ17-nervonic acid has also been reported (21).

The sphingolipid preparations analyzed in the present studies were mixtures of a number of complex lipids, and more work will be required to determine the distribution of fatty acids in better defined lipid classes. Preliminary studies on the distribution of fatty acids in human brain sphingolipid preparations have recently been reported by Schwarz *et al.* (22).

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