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Separation and isolation of methyl esters and dimethylacetals formed from brain lipids

L. F. Eng, Y.L.Lee, R. B. Hayman, and B. Gerstl

Research Laboratory, Veterans Administration Hospital, Palo Alto, California, and Department of Pathology, Stanford University, Stanford, California

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The procedure of Stoffel, Chu, and Ahrens (1) for preparing and separating the methyl esters of fatty acids and the dimethylacetals (DMA) when applied to brain lipid extracts proved adequate for the nonoxygenated fatty acids and DMA. Some of the cholesterol in the extract, however, was converted to derivatives, one of which, $\Delta^{3,5}$-cholestadiene (2), sublimed along with the methyl esters and the DMA. Furthermore, it was found that under the conditions specified by Stoffel et al. (1), approximately 75% of the 2-hydroxy methyl esters remained unsublimed.

Reports (3–5) on the fractionation and estimation of nonoxygenated and oxygenated fatty acid derivatives suggest that a thin-layer chromatographic procedure (TLC) could be developed to separate quantitatively the esters of the nonoxygenated and of the 2-hydroxy fatty acids as well as of the DMA. This consideration and the possibility that cholesterol and its derivatives could be separated were explored.

Chimyl alcohol, glyceryl monostearate, 12-hydroxy stearic acid, behenic acid, 2-hydroxy behenic acid, oleyl and behenyl alcohols, nonoxygenated methyl esters,[1]

[1] Abbreviations used: Fatty acids are identified by their carbon number and number of double bonds. An *h* indicates the presence of a 2-hydroxy group.

cholesterol and tetra- and octadecanal were obtained commercially. The 2-hydroxy methyl esters[1] C18h:0, C22h:0, and C24h:0, were a generous gift of Dr. J. F. Mead.

The procedures for the extraction of lipids and the determination of carboxyl esters and aldehydes have been reported previously (2). The fatty acids and DMA were methylated in sealed tubes according to Farquhar (6).

Chromatoplates, 20 × 20 cm, were coated with layers, 250–275 μ thick, of Silica Gel G (Research Specialties Co., Richmond, Calif.), using the Desaga-Brinkmann equipment (Brinkmann Instruments Co., Great Neck, N.Y.) according to Stahl (7). The plates were activated at 100° for 2 hr. The chromatographic chamber was lined with Whatman No. 1 filter paper and flushed once with nitrogen after the introduction of 200 ml of developing solvent. The solvents used were petroleum ether (bp 40–60°)–diethyl ether 60:40 (v/v) (8) and xylene, which were redistilled from an all-glass still within 24 hr prior to use. These solvents were allowed to equilibrate in the chamber for 1 hr before development of the chromatoplates. The entire chromatographic procedure was performed without direct exposure to light.

Gas-liquid chromatography (GLC) was done on a Barber-Coleman Model 10 apparatus equipped with a radium ionization detector as described elsewhere (2).

The chromatoplates were marked with the aid of the Brinkmann labeling template. The points of origin started 3 cm from the left edge and 2.5 cm from the base of the plate. Exactly 9 cm above the origin, a 5-mm line was drawn parallel to the base at the left and right edge of the plate. Fifteen centimeters above the origin, a line was drawn parallel to the base across the entire plate.

A solution of methylation products obtained from a brain lipid extract containing 10–20 μmoles of methyl esters was placed in a 13 x 100-mm test tube and concentrated to 0.2–0.3 ml with a stream of nitrogen. The entire sample was applied with a micropipette to a chromatoplate in a row of 30 spots, about 5 mm apart. The plate was first developed in the petroleum ether–diethyl ether mixture until the solvent front had ascended 9 cm above the origin. The plate was removed from the first solvent system, dried with a stream of nitrogen, and placed in a chamber containing xylene. After the xylene had migrated 15 cm above the origin, the plate was removed, partially dried with nitrogen, and allowed to dry in a hood for 30 min. The dried chromatoplate was placed for 5–10 sec in a glass chamber (20-liter volume containing 2 g of iodine crystals at its bottom) to render the zones of the various lipids visible. Immediately after exposure, the lipid bands were marked, and the plate was flushed with a stream of nitrogen to remove the iodine. Each band of lipid was scraped off quantitatively with a 1-cm segment of a razor blade and transferred to a 15 x 130-mm test tube with a Teflon-lined screw top. Ten milliliters of petroleum ether–diethyl ether 60:40 were added to the nonoxygenated methyl ester fraction, and 5 ml to the 2-hydroxy methyl ester and DMA fractions. Each tube was tightly capped and mixed three times by placing it on a "Vortex Jr. Mixer" (Scientific Industries Inc., Springfield, Mass.) for 15–30 sec and set aside in the dark for 30 min to allow the silica gel to settle. After suitable aliquots of each fraction were removed for quantitative analysis of carboxyl esters and aldehydes, the tubes were centrifuged for 5 min at 1,000 × *g*, and aliquots were removed for GLC.

Development with a solvent mixture of petroleum ether and diethyl ether separated distinctly the normal methyl esters and DMA from the 2-hydroxy methyl esters, long chain alcohols, cholesterol, and the major cholesterol derivative (Table 1). The methyl esters of the nonoxygenated fatty acids, however, were not separated from the DMA. While development with xylene did separate the nonoxygenated methyl esters from the DMA (Table 1), the 2-hydroxy methyl esters and cholesterol remained close to the origin. Consecutive development of the chromatoplate with petroleum ether–diethyl ether and xylene (Table 1) accomplished separation of the DMA, nonoxygenated methyl esters, and 2-hydroxy methyl esters from each other, and from cholesterol and its major derivatives. Some cholesterol derivatives, however, migrated to the same areas as the DMA, and the 2-hydroxy methyl esters were not separated from alcohols of similar chain lengths.

Employing the petroleum ether–diethyl ether or the xylene solvent system for quantities between 0.8 and 22 μmoles, the recoveries of the nonoxygenated methyl

TABLE 1 R_F VALUES OF VARIOUS LIPIDS ON CHROMATOPLATES

Compounds	Solvent System A*	B†	C‡
		R_F§	
Nonoxygenated methyl esters	0.78	0.43	0.64
2-Hydroxy methyl esters	0.41	0.05	0.28
12-Hydroxy methyl stearate	0.41	0.05	0.26
Dimethylacetals	0.78	0.15	0.53
Cholesterol	0.20	0.05	0.15
Cholestadiene	0.90	0.83	0.83
Glyceryl monostearate	0.03	0.00	0.03
Chimyl alcohol	0.03	0.00	0.03
Oleyl alcohol	0.40	0.05	0.26
Behenyl alcohol	0.40	0.05	0.26

* Petroleum ether–diethyl ether 60:40 (v/v).
† Xylene.
‡ Consecutive development with petroleum ether–diethyl ether and xylene.
§ R_F values given for each solvent system were determined on a single plate.

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Table 2 Recoveries of a Synthetic Mixture of Purified Non-Oxygenated Methyl Esters, 2-Hydroxy Methyl Esters, and Dimethylacetals from Thin-Layer Chromatography

Sample	Nonoxygenated Methyl Esters*		2-Hydroxy Methyl Esters†		Dimethylacetals‡		Percentage of 2-Hydroxy Methyl Esters in the Mixture	
	μmoles Applied	% Recovery	μmoles Applied	% Recovery	μmoles Applied	% Recovery	Original	Found
Plate 1	9.5	105	1.56	90	1.42	92	14.1	12.4
Plate 2	9.5	102	1.56	93	1.42	90	14.1	13.0
Plate 3	9.5	102	1.56	89	1.42	96	14.1	12.6
Plate 4	9.5	102	1.56	107	1.42	94	14.1	14.8
Average		103		95		93		13.2

* Mixture of chromatographically purified C16:0, C18:0, C22:0, C20:5, and C22:6 methyl esters.
† Mixture of chromatographically purified C18h:0, C22h:0, and C24h:0 2-hydroxy methyl esters.
‡ Mixture of chromatographically purified dimethylacetals prepared from brain lipids.

esters ranged from 97 to 99%, that of the oxygenated methyl esters (C22h:0 and C24h:0) ranged from 94 to 98%. After consecutive development with these solvent systems, the recoveries of the individual nonoxygenated methyl esters were 99% for C18:1, C20:1, and C24:1; 94–99% for C22:5; and 92–99% for C22:6. A recovery of 99% was obtained for a mixture of C18:1, C20:1, C20:5, C22:6, and C24:1. Analysis of this mixture by GLC before and after TLC showed an essentially identical percentage composition. The recoveries of the individual 2-hydroxy methyl esters or that of their mixture (C18h:0, C22h:0, and C24h:0) varied from 88 to 94%. While Vioque and Holman (5) reported that exposure to iodine vapors caused some loss of the unsaturated fatty acids, it is evident from the preceding data that "brief" exposure to iodine vapors does not destroy appreciable amounts of any of the unsaturated fatty acids examined.

The recovery of DMA of tetradecanal and octadecanal from TLC ranged between 95 and 98%, and that of the DMA from brain lipids purified by sublimation according to Farquhar (6) was 99%. Recovery of the DMA from unsublimed methylation products varied from 74 to 99%. It averaged 85% for 33 plates, and its reproducibility on any particular lipid sample was generally within 5%; thus, lower recovery was characteristic of certain lipid samples rather than of the TLC procedure. Reasons for low recovery of DMA from these extracts have not been ascertained as yet.

Recoveries from a synthetic mixture of purified methyl esters and DMA (Table 2) on four plates were 102–105% for the nonoxygenated esters, 89–107% for the 2-hydroxy methyl esters, and 90–96% for the DMA. Recoveries of methyl esters from brain lipid extracts on 37 plates varied between 89 and 102%, with an average of 99%. In one sample, an unexplained recovery of 114% was noted. The percentage deviation for replicate analyses of 2-hydroxy fatty acids was 2.4%. These data were based upon 14 duplicates, 3 triplicates, 1 quadruplicate, and 2 quintuplicate experiments.

The procedure presented is simple, rapid, and applicable for studying the fatty acids from brain and possibly other tissue lipid extracts. The results suggest that the DMA can be reliably analyzed by GLC if the steroids are removed from the lipid extract prior to methylation.

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