notes on methodology

Direct transesterification of lipids in mammalian tissue for fatty acid analysis via dehydration with 2,2′-dimethoxypropane

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Summary A method is described for the transesterification of lipids of mammalian tissues for fatty acid analysis by gas–liquid chromatography (GLC) that eliminates the extraction step of conventional procedures. The method involves the direct reaction of anhydrous HCl–methanol with the lipids in approximately 10 mg of tissue or 0.1 ml of serum after removal of water by reacting it with 2,2′-dimethoxypropane (DMP). Acetone and methanol produced from water in the sample, as well as the excess DMP, are evaporated prior to transesterification in order to eliminate the formation of artifacts from the solvents. The method was demonstrated with rat serum and brain tissue.

Supplementary key words rat serum · rat brain tissue

Generally the lipid of animal tissues and serum is extracted and converted to methyl esters by transesterification with methanol by one of several standard procedures (1) for determination of fatty acid composition by GLC. Reported here is a simple procedure that eliminates the extraction step by dehydration of the sample with DMP. Evaporation of the excess DMP, as well as the acetone and methanol to which water is converted, permits application of the transesterification reaction directly to tissue and serum.

Materials and methods

DMP, Eastman Organic Chemicals (Rochester, NY), was purified by distillation over potassium hydroxide pellets and saturated with nitrogen. Anhydrous HCl–methanol was prepared by bubbling dry HCl into anhydrous, freshly distilled methanol until the concentration was 5% by weight of the solution. Brain tissue and serum of male Sprague-Dawley rats (approximately 175 g) fed Purina Lab Chow were used to demonstrate the procedure.

Methyl esters were prepared as follows. Approximately 10 mg of tissue or 0.1 ml of serum was placed in a Pyrex test tube (10 × 125 mm) with one ml of DMP containing 20 μl of concentrated HCl. The tube was flushed with nitrogen to minimize chances of autoxidation and shaken gently for approximately 20 min to facilitate the reaction of DMP with water. Acetone and methanol produced in the reaction and the excess DMP were evaporated in a stream of nitrogen at room temperature. The residue was mixed with 2.5 ml of the anhydrous HCl–methanol reagent and heated under an atmosphere of nitrogen in the same tube sealed with a Teflon-lined cap at 80–85°C in a water bath.

For tissues such as liver, with lipids mostly of the *O*-acyl type, a 1-hr reaction time was adequate. For most tissues containing lipids with relatively large amounts of sphingolipids, a 4-hr reaction time appeared to be adequate but, with some tissues, it might be necessary to carry out the reaction in a sealed Pyrex tube under an atmosphere of nitrogen in a boiling water bath at 95°C (2–4) to insure complete transesterification of all of the *n*-acyl-linked fatty acids.

After completion of the transesterification of the fatty acids, an equal volume of distilled water was added to the reaction mixture and the methyl esters were extracted into low boiling petroleum ether with two 5-ml extractions. The petroleum ether extracts were combined, dried over anhydrous sodium sulfate, and filtered; the crude methyl esters were recovered by evaporation of the solvent. The methyl esters were dissolved in 50 μl of carbon disulfide for GLC analysis.

The method was demonstrated with rat serum and brain tissue. For comparison, the fatty acid composition was also determined by conventional procedures on the lipid of these samples extracted with chloroform–methanol 2:1 (v/v), using the anhydrous HCl–methanol reagent as previously described (2–4) and conc. HCl–methanol as described by Gaver and Sweeley (5).

GLC was carried out with a Barber Coleman gas chromatograph equipped with a flame ionization detector and a 6′ × ¼″ glass column packed with 15% EGS on Gas-Chrom P, 100–200 mesh (Applied Science Lab, Inc., State College, PA) at 180°C. Nitrogen was used as the carrier gas at a flow rate of 30

Abbreviations: GLC, gas–liquid chromatography; DMP, 2,2′-dimethoxypropane; TLC, thin-layer chromatography.

ml/min and the percentage composition was calculated from the proportionalities of the peak areas by an automatic digital integrator (Model CS 1-208, Columbia Scientific Industries, Austin, TX).

Thin-layer chromatography (TLC) was carried out with plates coated with Silica Gel G (Merck A.G., Darmstadt, Germany) and activated at 110°C for 1 hr.

Results

Chromatograms of the methyl esters obtained from rat liver tissue by transesterification with anhydrous HCl–methanol, before and after evaporation of both the DMP and the acetone formed in the reaction with water in the tissue, are shown in **Fig. 1**. Comparison of the chromatograms (Figs. 1A and 1B) show that the artifacts from these solvents had retention times approximating those of short chain saturated methyl esters on a polar stationary phase (Fig. 1A). When the reaction was carried out after removal of the DMP and acetone, artifacts from these solvents were completely eliminated (Fig. 1B).

Thin-layer chromatograms of the petroleum ether extract of the transesterification reaction mixture obtained from brain tissue are shown in **Fig. 2**. These analyses showed that, in addition to methyl esters, the petroleum ether extracts contained cholesterol, small amounts of free fatty acids, and two other components. The component near the solvent front on the plate (X_1, Fig. 2) was an artifact produced from cholesterol. This artifact was produced in larger amounts from the conc. HCl–methanol reagent than from anhydrous HCl–methanol. The other component that separated above cholesterol (X_2, Fig. 2)

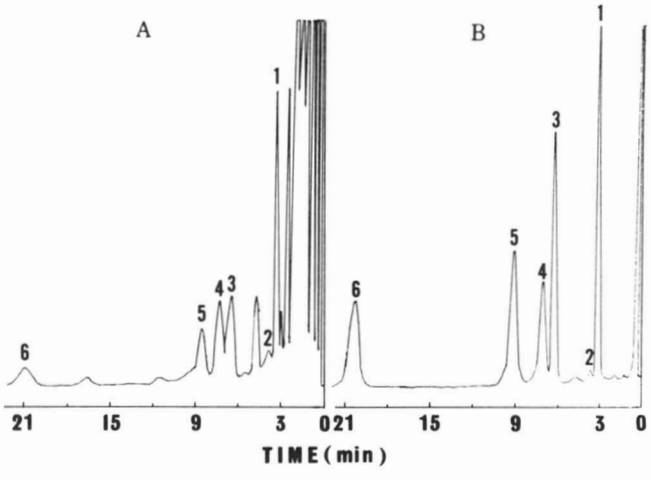

Fig. 1. GLC showing artifact formation from 2,2′-dimethoxypropane (DMP). *A*. Before removal of DMP, and *B*, after removal of DMP in the analysis of the fatty acids in liver tissue via transesterification with anhydrous HCl–methanol. 1 = 16:0, 2 = 16:1, 3 = 18:0, 4 = 18:1, 5 = 18:2, 6 = 20:4.

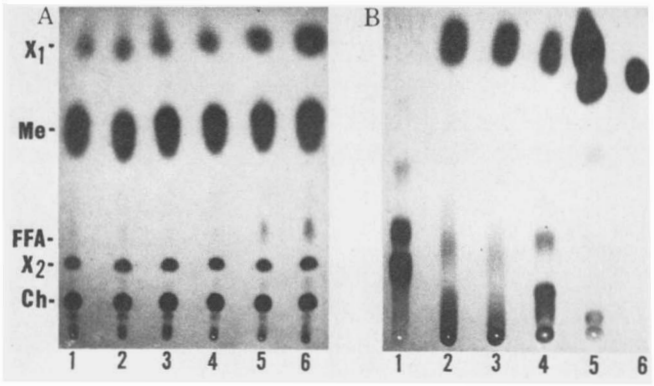

Fig. 2. *A*, TLC: Brain tissue lipids after transesterification. *1* and *2*, DMP anhydrous HCL–methanol method at 85°C, 2 and 4 hr reaction times, respectively; *3* and *4*, anhydrous HCl–methanol at 85°C, 2 and 4 hr reaction times on extracted lipid, respectively; *5* and *6*, 2 and 5 hr reaction times at 85°C respectively on extracted lipid with conc. HCl–methanol. X_1, cholesterol artifact; Me, methyl ester; FFA, free fatty acid; X_2, unidentified; CH, cholesterol. Solvent system, petroleum ether–ethyl ether–acetic acid 85:15:1. *B*, TLC: *1*, Ceramides (Supelco Inc., Bellefonte, PA); *2* and *3*, ceramides after transesterification with anhydrous HCl–methanol 2 and 4 hrs at 85°C, respectively; *4*, ceramides after transesterification for 5 hr with conc. HCl–methanol at 85°C; *5*, rat brain extract after transesterification by DMP, anhydrous HCl–methanol method for 4 hr at 85°C; *6*, cholesterol. Solvent system, chloroform–methanol–ammonium hydroxide 90:10:1.

was not identified but was believed to be an artifact of the glycosphingolipids, possibly the *O*-methyl ether of long chain bases (6–8) inasmuch as it was not detected among the products of the transesterification of serum or liver lipids. No ceramide was detected among the products of the transesterification of brain lipids either with the DMP–anhydrous HCl–methanol procedure or with the conc. HCl–methanol in the procedure of Gaver and Sweeley (5) which was applied directly to the extracted lipid, as also illustrated in Fig. 2.

Free fatty acids were readily detected among the products of the reaction in the conc. HCl–methanol method, but were not present in significant amounts among the products of reaction when anhydrous HCl–methanol was used as the transesterification reagent on either the tissue or the extracted lipid.

Determination of the fatty acid composition of brain tissue by the DMP–HCl method showed that the analyses were essentially unchanged after a reaction period of 1 hr as shown in **Table 1**. Moreover, the results with the DMP method were essentially identical to those obtained with the conc. HCl–methanol method that was applied directly to the extracted lipid (Table 1). Peaks 2, 5, and 6 are not calculated in the results because they are not methyl esters but acetals derived from plasmalogens by the action of HCl and methanol. Rat brain lipids contain a number of fatty acids in trace and minor amounts in

TABLE 1. Fatty acid analyses of rat brain (transesterification with methanol)[a]

Peak No.	Fatty[b] Acid	DMP Method[c]				Anhydrous HCl[d]		Conc. HCl[e]	
		1 hr	2 hr	4 hr	24 hr	2 hr	4 hr	2 hr	5 hr
		percentage of total fatty acids							
1	14:0	trace	trace	trace	trace	trace	trace	trace	trace
3	16:0	17.4 ± 0.2	17.1 ± 0.1	17.1 ± 0.4	18.1 ± 0.4	19.4 ± 0.2	19.0 ± 0.5	19.5 ± 0.2	19.2 ± 0.3
4	16:1	0.4 ± 0.2	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.1
7	18:0	24.6 ± 0.2	24.4 ± 0.3	24.6 ± 0.5	23.9 ± 0.5	24.6 ± 0.3	25.1 ± 0.1	24.9 ± 0.4	25.1 ± 0.2
8	18:1	22.3 ± 0.4	21.3 ± 0.1	21.3 ± 1.0	21.4 ± 0.3	20.4 ± 0.5	20.5 ± 0.3	20.3 ± 0.2	19.9 ± 0.1
9	18:2	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
10	20:0	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
11	20:1	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.0 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.6 ± 0.1
12	20:2	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	trace	trace	0.3 ± 0.2	0.3 ± 0.1
13	20:3	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	trace	0.2 ± 0.1	0.2 ± 0.1	trace
14	20:4	10.5 ± 0.1	10.0 ± 0.2	10.2 ± 0.3	10.6 ± 0.5	11.0 ± 0.2	11.3 ± 0.2	10.8 ± 0.1	10.9 ± 0.2
15	20:5	0.3 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
16	22:4 (n − 6)	4.1 ± 0.1	4.3 ± 0.4	4.4 ± 0.2	4.2 ± 0.5	4.3 ± 0.2	4.3 ± 0.1	4.2 ± 0.2	4.5 ± 0.4
17	22:5 (n − 6)	1.9 ± 0.1	2.0 ± 0.1	2.4 ± 0.4	1.7 ± 0.2	1.7 ± 0.2	1.6 ± 0.2	1.9 ± 0.2	2.1 ± 0.3
18	22:6	14.7 ± 0.9	15.9 ± 0.6	15.1 ± 0.3	15.7 ± 0.4	14.9 ± 1.0	14.4 ± 0.5	14.7 ± 1.0	14.7 ± 0.3

[a] Mean ± SD, samples from three rats of same group, trace = <0.2%.
[b] Identification based on relative retention times; acetals from aldehydes or ether lipids not reported, peaks no. 2, 5, and 6.
[c] See text for details.
[d] At 85°C (See Ref. 4).
[e] At 85°C (See Ref. 5).

addition to those reported here. In order to detect these, it should be necessary to apply methods of methyl ester fractionation in conjunction with more sophisticated methods of GLC. Nevertheless, the fact that some minor constituent fatty acids were detected, as well as methyl docosahexaenoate (Table 1), indicated further that all of the fatty acids underwent transesterification.

Only the major fatty acids were determined in the application of the DMP–anhydrous HCl–methanol to rat serum (**Table 2**). However, the results agreed closely with those obtained by the application of conventional methods directly to the extracted lipids.

Discussion

DMP is commonly used as a water scavenger and has been employed to drive the esterification of lipids to completion (9). However, its direct use in an esterification reaction with HCl–methanol results in the formation of polymeric artifacts that interfere with the GLC analysis of methyl esters of fatty acids, as shown herein and as demonstrated by Simmonds and Zlatkis (10). The formation of artifacts from DMP and acetone produced from water may be minimized by the use of inhibitors of polymer formation (10) or by conducting the reaction overnight at low temperatures (9, 11). However, neither of these techniques offer any advantages over more conventional transesterification methods used for the preparation of methyl esters in determinations of the fatty acid composition of tissue lipids. In the use of DMP to insure anhydrous conditions, the problem of artifact formation is eliminated in the procedure reported here by the simple expediency of removing the

TABLE 2. Comparison of fatty acid analyses of rat serum[a] via DMP anhydrous HCl–methanol method[b] and transesterification of extracted lipid with anhydrous HCl–methanol at 85°C

Fatty Acids (Major Components)	DMP Method	Anhydrous HCl–Methanol
	percentage of total fatty acids	
16:0	19.5 ± 0.6	20.0 ± 0.7
16:1	2.5 ± 0.4	2.6 ± 0.3
18:0	8.7 ± 0.9	8.4 ± 1.0
18:1	14.4 ± 1.1	14.9 ± 1.0
18:2	22.1 ± 2.0	21.9 ± 2.3
20:4	28.1 ± 3.0	27.4 ± 2.7

[a] Mean ± SD, analysis of serum from five rats of the same group.
[b] DMP, dimethoxypropane method. See text for details.

excess DMP, as well as the acetone that is produced from water, by evaporating them prior to transesterification with anhydrous HCl–methanol.

It has been reported (12, 13) that transesterification of *n*-acyl-linked fatty acids in sphingolipids does not go to completion with anhydrous HCl–methanol reagents. However, the conditions of transesterification used in these studies were much milder than those employed here. The method of transesterification used in conjunction with DMP for dehydration of tissue appeared to give complete reaction inasmuch as no ceramide was detected among the products of the reaction. In spite of the more vigorous conditions employed with anhydrous HCl–methanol, artifact formation did not appear to be any greater with this reagent than with the conc. HCl reagent. In fact, less artifact appeared to be formed from cholesterol with the anhydrous HCl than with the conc. HCl reagent; however, as demonstrated by Kramer and Hulan (14), alkali-catalyzed hydrolysis is preferred for the preparation of free sterols. According to Christie (1), artifact formation is increased in methods using anhydrous HCl reagents when care is not taken to vigorously exclude oxygen during the reaction. The artifacts produced in the application of the method to rat brain tissue did not appear to interfere with the GLC analysis; however, interference of artifacts can be readily eliminated by purification of the methyl esters by TLC.

Significant amounts of free fatty acids are produced from aqueous HCl reagents because the reaction is reversible and, water being a stronger electron donor than aliphatic alcohols, favors the formation of free fatty acids. Moreover, unlike anhydrous methanol, which forces the transesterification to completion, an excess of an aqueous reagent has no such effect. For this reason, anhydrous HCl–methanol used as described here and as reported previously appears to be more desirable for the transesterification of lipids than conc. HCl–methanol. Moreover, a high temperature–short reaction time appears to be more desirable than a long reaction time at low temperature because the reaction with the glycosphingolipids is complete and less artifacts that might interfere with the GLC analysis are formed.

No studies were made on the use of the DMP anhydrous HCl–methanol technique for analysis of the long chain bases of glycosphingolipids because such a study is beyond the scope of the present work. However TLC of the reaction products remaining in the aqueous phase after extraction with petroleum ether did not appear to differ qualitatively or quantitatively from those produced with conc. HCl–methanol by the method of Gaver and Sweeley (5).

The procedure described here provides a simplification of conventional methods for the determination of fatty acid composition of tissue and serum and is ideally suited to routine analyses of multiple samples. Whether it can also facilitate the analysis of long chain bases will have to await further investigation.

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