A simple procedure for rapid transmethylation of glycerolipids and cholesteryl esters

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Summary A simple procedure suitable for rapid transmethylation of triacylglycerols, other neutral lipids (including cholesteryl esters), and glycerophospholipids is described. Lipids in diethyl ether solution (50 volumes), in the presence of methyl acetate (1 vol), are reacted with 1M sodium methoxide in methanol (1 vol) at room temperature. Essentially complete transmethylation can occur within a few minutes with no hydrolysis. Glassware and reagent requirements are minimal and samples are ready for gas–liquid chromatography analysis with very little work-up.—**Christie, W. W.** A simple procedure for rapid transmethylation of glycerolipids and cholesteryl esters. *J. Lipid Res.* 1982. **23:** 1072–1075.

Supplementary key words triacylglycerols • glycerophospholipids

Innumerable procedures have been described for transmethylation of glycerolipids, prior to gas–liquid chromatographic (GLC) analysis of the fatty acid methyl esters, and they have been comprehensively reviewed (1–3). These methods are frequently needlessly complex, use large volumes of reagents, and have various washing steps, during which contaminants can be introduced and losses can occur. Milk fat triacylglycerols in hexane solution (19 volumes) were shown to be transesterified rapidly by means of sodium methoxide in methanol (1 volume) (4). No washing or solvent-removal steps were required prior to GLC analysis, but small amounts of sodium methoxide injected onto the GLC column were found to produce spurious peaks in some circumstances (5, 6). A method based on this principal has now been developed that is suited to a wide range of lipid classes. Diethyl ether is used to solubilize polar lipids, methyl acetate is added to minimize competing

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

irreversible hydrolysis reactions, and the mixture is neutralized with acid when the reaction is complete. Small volumes only of reagents are used, and the method has a minimum of work-up steps.

MATERIALS AND METHODS

Methyl [1-^{14}C]palmitate, cholesteryl [1-^{14}C]palmitate, and [1-^{14}C]dipalmitoylphosphatidylcholine (about 20,000 dpm per mg) were synthesized by acylation of methanol, cholesterol, and palmitoyllysophosphatidylcholine (Sigma (London) Chemical Co., Poole, Dorset), respectively, with the acid chloride derivative of [1-^{14}C]palmitic acid (Amersham International, Amersham, Bucks.) (7). They were purified by preparative thin-layer chromatography (TLC) before use. All reagents were Analar grade (Fisons Ltd., Loughborough, Leics.).

Recommended transmethylation procedure

Glycerolipids (1–10 mg) are dissolved in sodium-dried diethyl ether (1 ml) and methyl acetate (20 μl). Then 1 M sodium methoxide in dry methanol (20 μl) is added, and the solution is agitated briefly to ensure thorough mixing. The solution immediately becomes cloudy as sodium-glycerol derivatives are precipitated. After 5 min at room temperature, the reaction is stopped by adding a saturated solution of oxalic acid in diethyl ether (30 μl) with brief agitation. The mixture is centrifuged at about 1500 *g* for 2 min to precipitate sodium oxalate, and the solvent is removed in a gentle stream of nitrogen at room temperature (taking care not to blow out the precipitate). Fresh diethyl ether (1 ml) or hexane is added, and an aliquot of this is taken directly for GLC analysis.

For smaller amounts of glycerolipids (<1 mg), the volumes of the reagents should be reduced proportionately. A longer reaction time (1 hour) is required for cholesteryl esters. If shorter-chain (C_{12} and below) esters are present, the solvent-evaporation step should be avoided.

Separation of isotopically labeled products of transmethylation reactions

Lipids were separated by preparative TLC on silica gel G layers (0.5 mm thick; E. Merck, Darmstadt), with unlabeled lipids added as carriers, and were visualized under ultraviolet light after spraying with 2′,7′-dichlorofluorescein solution (2). Bands were scraped into scintillation vials and were suspended in Insta-Gel® (Packard Instrument Co., Caversham, Berks.) and water (7:3 by vol) for liquid scintillation counting. With cholesteryl ester transmethylations, for example, hexane–diethyl ether–formic acid 90:10:1 (by vol) was the solvent for TLC development, and cholesteryl ester, methyl ester, and free fatty acid bands were collected.

TABLE 1. Hydrolysis (%) of methyl [1-^{14}C]palmitate by sodium methoxide in diethyl ether in the presence of methyl acetate

	Volume of Methyl Acetate						
Wt of Lipid	0	5	10	15	20	40	80
mg				*µl*			
5	10.2	9.6	5.1	3.8	1.0	1.0	0.5
0.5	98.5	75.5	52.0	17.1	1.0	1.2	0.5

Methyl [1-^{14}C]palmitate was dissolved in diethyl ether (1 ml) and methyl acetate (volumes indicated), and 1 M sodium methoxide in methanol (20 μl) was added. After brief agitation, the solutions were left at room temperature for 5 min, when the reaction was stopped by addition of acetic acid (5 μl). Products (free fatty acids and unchanged methyl palmitate) were separated by TLC for liquid scintillation counting.

GLC analysis of methyl esters

Methyl esters were separated on a 3 m × 2 mm glass column packed with Silar 10C® on Gas-Chrom Q® (Applied Science Laboratories, State College, PA), used isothermally at 195°C with argon (12 ml/min) as carrier gas, in a Pye GCD gas chromatograph (Pye-Unicam, Cambridge). Components were quantified by electronic integration.

RESULTS

In preliminary experiments, in which the method of Christopherson and Glass (4) was used to transesterify a variety of polar lipids (but with diethyl ether as solvent), it was observed that the methyl esters formed were rapidly hydrolyzed. Presumably trace amounts of water taken up from the atmosphere or adsorbed on glassware generated sufficient sodium hydroxide to bring about irreversible hydrolysis (1). The magnitude of the effect appeared to depend on the amount of lipid present to some extent.

In an attempt to minimize the problem, some experiments were carried out with increasing amounts of methyl acetate in the medium, in the hope that as there would be high molar proportions of this relative to lipids, the methyl acetate would be hydrolyzed preferentially. Methyl [1-^{14}C]palmitate in diethyl ether (50 vol) was reacted with 1 M sodium methoxide in methanol (1 vol) for 5 min at room temperature, with various amounts of methyl acetate present. (Isotopically labeled lipids were used initially as test substances for the reaction). The products (methyl esters and free fatty acids) were separated by TLC for liquid scintillation counting and the results are shown in **Table 1**. With higher amounts of substrate, the amount of hydrolysis obtained

TABLE 2. Transmethylation of cholesteryl [1-^{14}C]palmitate, glycerol tri-[1-^{14}C]palmitate and di-[1-^{14}C]palmitoylphosphatidylcholine with respect to time; results expressed as percentage of starting material transesterified

	Time				
	2	5	10	20	40
	min				
Cholesteryl palmitate	55.8	77.5	87.7	93.8	96.2
Tripalmitin	99.4	99.5	99.5	99.5	99.5
Dipalmitoylphosphatidylcholine	96.7	97.9	98.0	98.1	97.9

Reaction conditions as in the footnote to Table 1 but with 20 μl methyl acetate. Five mg of each lipid was used. Methyl esters and unchanged starting materials were separated by TLC for liquid scintillation counting.

in the absence of methyl acetate was relatively small, but not negligible. With small amounts of substrate, hydrolysis was almost complete in the absence of methyl acetate, but dropped to negligible proportions as methyl acetate was added.

The same volumes of methyl acetate and sodium methoxide-methanol were used in all subsequent work, as this gave the maximum rate of transesterification compatible with minimum hydrolysis. The combined volumes of the reagents were reduced for small amounts of lipid. Diethyl ether was retained as the main solvent, as it is easily removed by evaporation, but other solvents gave satisfactory results.

Various lipid classes were transesterified in diethyl ether (50 vol) and methyl acetate (1 vol) with sodium methoxide in methanol (1 vol) at room temperature, and the reaction was stopped at regular intervals so that the products of the reaction could be isolated for analysis. The results are shown in **Table 2**. Dipalmitoylphosphatidylcholine and tripalmitin were essentially transesterified in 2–5 min; cholesteryl esters reacted more slowly, as has been found with other transesterification procedures (2), but satisfactory results were obtained after 1 hr of reaction. In none of these experiments was there any significant accumulation of free fatty acids (>0.1%). Analogous results were obtained when the reaction was repeated on the 0.1 mg scale.

During transmethylation of cholesteryl palmitate in the presence of methyl acetate, a small amount of cholesteryl acetate produced by base-catalyzed exchange was found, but this did not interfere with GLC analyses in practice. Base-catalyzed transesterification procedures do not produce other artefacts that can interfere with analysis (2).

When transmethylation is complete, it is advisable to acidify the reaction mixture to prevent hydrolysis occurring during extended periods of storage. A precipitate of the sodium salt is formed. Following centrifugation, when an aliquot of the supernatant liquid was injected onto the Silar 10C GLC column in routine use in this laboratory, a spurious unsymmetrical peak with a sharp leading edge was obtained in every case just ahead of methyl octanoate, due to reaction of excess acid in solution on the stationary phase. The top few centimeters of the column packing may deteriorate slowly but this is easily replaced if necessary. Spurious acid peaks were virtually eliminated by using a non-volatile organic acid of comparatively low solubility, e.g., oxalic, evaporating off all the solvent, especially the methanol, following centrifugation and adding back diethyl ether alone as solvent for injection onto the GLC column.

DISCUSSION

The procedure described here has been used in this laboratory for some time with a wide variety of samples. In comparison with other methods that require longer reaction times and extensive manipulations, the new procedure has been found to have superior reproducibility, especially when polyunsaturated components are present. No significant difference in reproducibility was observed, whether the solvents were evaporated, following acidification and centrifugation, and replaced by fresh diethyl ether, or whether an aliquot of the supernatant was injected directly onto the GLC column. It is recommended that the latter approach be used if shorter-chain (C_{12} or less) esters are believed to be present, although a wider solvent peak and an acid-artefact peak are obtained, and the recorder base-line takes longer to return to normal. If shorter-chain esters are not present, excess reagents should be evaporated and replaced by fresh solvent, to eliminate artefact peaks and to prolong the life of the column.

The procedure effects a rapid and quantitative transmethylation of ester bonds to glycerol or cholesterol. There is a minimal glassware requirement and the volumes of solvent and reagents used are small, limiting the chances of accidental contamination of samples. Methyl esters are ready for analysis within 5–10 min of commencing the reaction. The method could not be adapted for use in the presence of TLC adsorbents as excessive hydrolysis occurred, nor can any sodium methoxide-catalyzed transmethylation be used for free fatty acids or amide-bound fatty acids (1, 2).

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