Transesterification of phospholipids or triglycerides to fatty acid benzyl esters with simultaneous methylation of free fatty acids for gas-liquid chromatographic analysis

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Summary A novel method is presented for transesterification of fatty acid esters in phospholipids and triglycerides to benzyl esters while simultaneously recovering free fatty acids as methyl esters. Transesterification is catalyzed by 0.2 M (m-trifluoromethyl phenyl)trimethyl ammonium hydroxide in methylene chloride, 10% (v/v) benzyl alcohol, and 1% (w/v) potassium *tert*-butoxide, and is complete in 30 min at room temperature. Methyl esters of all common fatty acids separate from the benzyl esters formed from phospholipids. This method has broad utility and is applicable to the formation of esters optimized for detection by absorbance or fluorescence (high performance liquid chromatography), electron capture (gas-liquid chromatography), or negative ion chemical ionization (gas-liquid chromatography-mass spectrometry). — **van Kuijk, F. J. G. M., D. W. Thomas, J. P. Konopelski, and E. A. Dratz.** Transesterification of phospholipids or triglycerides to fatty acid benzyl esters with simultaneous methylation of free fatty acids for gas-liquid chromatographic analysis. *J. Lipid Res.* 1986. **27:** 452-456.

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The fatty acid content of phospholipids or other glycerides is most frequently determined by transesterification to form fatty acid methyl esters (1, 2) followed by gas-liquid chromatography (GLC) (3). There are several instances where formation of different esters of the fatty

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Abbreviations: HPLC, high performance liquid chromatography; BHT, butylated hydroxy toluene; TLC, thin-layer chromatography; EDTA, ethylenediamenetetraacetate; GLC-MS, Gas-liquid chromatography-mass spectrometry.

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acids might be desirable. Benzyl esters have been used to decrease the volatility of shorter chain fatty acids for quantitative gas-liquid chromatographic analysis (4). Benzyl esters are also useful because of their ultraviolet absorbance which facilitates analysis by high performance liquid chromatography. HPLC may be preferred to gas-liquid chromatography if recovery of the fatty acids is required for determination of radioactivity or for some other purpose.

Current methods for the formation of fatty acid benzyl esters require free fatty acids and the use of benzyl bromide (2, 4). Therefore, if phospholipids or other glycerides are to be analyzed, the current methods require treatment with a lipase enzyme or a vigorous saponification step. Additional purification of the derivatives is then required before GLC. In this report we present a rapid method to obtain fatty acid benzyl esters by direct transesterification of phospholipids or other glycerides using benzyl alcohol in methylene chloride with a quaternary ammonium base and potassium *tert*-butoxide as catalysts. A novel feature of this method is that it distinguishes and simultaneously analyzes the free fatty acids in the sample as the methyl esters, while the fatty acids bound in phospholipids or other esters are recovered as the benzyl esters. The free fatty acids form quarternary ammonium salts which quantitatively decompose to methyl esters in the gas chromatograph injector (5, 6). This simultaneous, one-step determination recovers larger amounts of the most highly unsaturated free fatty acids in membranes by avoiding the TLC purification step usually employed that tends to oxidize these fatty acids. Other alcohols can be substituted for benzyl alcohol to generate derivatives optimized for detection by absorbance or fluorescence HPLC, electron capture GLC, or negative ion chemical ionization GLC-MS.

MATERIALS AND METHODS

Reagents

Di-heptadecanoyl-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine were obtained from Sigma Chemical Company (St. Louis, MO). Methanolic (m-trifluoromethyl phenyl) trimethyl ammonium hydroxide (0.2 M) (called Methyl Prep II) was a product of Applied Sciences Laboratories, Inc. (State College, PA). Benzyl alcohol was analytical grade (Mallinckrodt). Potassium *tert*-butoxide (Aldrich) was sublimed before use (7).

Gas-liquid chromatography

Gas-liquid chromatographic analyses were performed with a Perkin-Elmer model 3920 gas chromatograph with an all-glass sample stream, a 6 ft × 4 mm glass column containing 3% SP 2100 on Supelcoport, a nitrogen flow rate of 20 ml/min, and a flame ionization detector. The temperature was programmed from 175 to 250°C at 4°C/min. Injector and detector temperatures were 250°C. A Spectra-physics minigrator was used to establish retention times and peak areas.

Transesterification of phospholipids and triglycerides

Methanol was removed from 0.1 ml of a 0.2 M methanolic solution of (m-trifluoromethyl phenyl) trimethyl ammonium hydroxide by rotary vacuum evaporation and the sample was kept under vacuum for 10 min after it reached dryness to remove the last traces of methanol. The dry film of the ammonium salt was dissolved in 50 μl of benzyl alcohol and 50 μl of dichloromethane, and ca. 1 mg of sublimed *tert*-butoxide was added. This mixture is called the benzyl transesterification reagent. Samples of phospholipid or triglyceride (up to 500 μg) in 80 μl of dichloromethane, containing 50 μg of BHT/ml, were transferred to a glass vial. A 20-μl aliquot of the benzyl transesterification reagent was added, the vial was closed with a Teflon-lined screw cap and shaken to mix the contents. Samples were incubated at room temperature for 30 min. Fatty acid methyl esters were prepared from identical solutions in dichloromethane by adding 20 μl of Methyl Prep II.

Samples were analyzed by GLC as described above and were also spotted on silica gel TLC plates. The plates were developed in chloroform-methanol-water 65:35:5 (v/v/v) for phospholipids or in chloroform for triglycerides, and visualized by staining with iodine to confirm the completion of the transesterification reaction. Bovine retinal rod outer segments were isolated and purified by the method of Stone, Farnsworth, and Dratz (8). Lipids were extracted by the method of Bligh and Dyer (9), modified to use dichloromethane rather than chloroform (10). Methanol contained 50 μg/ml of butylated hydroxytoluene, and water contained 2 mM EDTA, pH 7.0. The dichloromethane phase containing phospholipids and free fatty acids was dried over sodium sulfate, the sample was removed from the drying agent, and the solvent was completely evaporated under nitrogen. The lipid film was dissolved in 80 μl of dichloromethane and 20 μl of the benzyl transesterification reagent was added.

RESULTS

Fig. 1A shows a chromatogram of the fatty acid methyl esters prepared from di(17:0)phosphatidylcholine and (16:0)(18:2)phosphatidylcholine. Fig. 1B shows the fatty acid benzyl esters made from the same phospholipid mixture and run with the identical temperature program. The percentage fatty acid composition of the phospho-

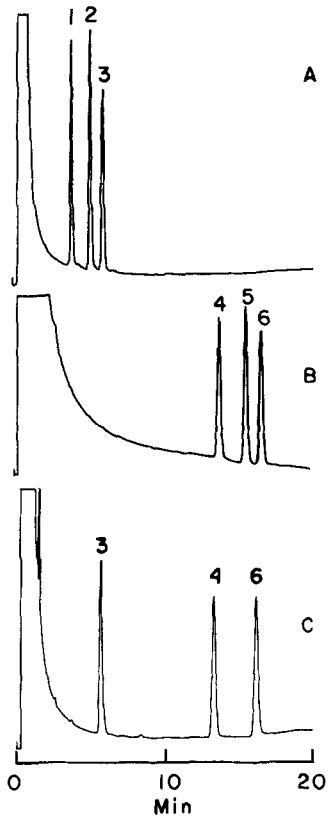

Fig. 1. A: Methyl esters of (16:0), (17:0), and (18:2) are peaks 1–3, respectively, separated by GLC on a 3% SP 2100 column programming the temperature from 175 to 250°C, at 4°C/min. The methyl esters were formed from di (17:0) phosphatidylcholine and (16:0)(18:2) phosphatidylcholine using Methyl Prep II. B: Benzyl esters of (16:0), (17:0), and (18:2) are peaks 4–6, respectively, separated by GLC under the same conditions as used for Fig. 1A. The benzyl esters were formed from di (17:0) phosphatidylcholine and (16:0)(18:2) phosphatidylcholine using the benzyl transesterification reagent as described in the text. C: Simultaneous analysis of free linoleic acid as the methyl ester derivative (peak 3) and (16:0)(18:2) phosphatidylcholine as the benzyl ester derivatives (peaks 4 and 6) using the benzyl transesterification reagent. The GLC conditions were the same as in Fig. 1A.

lipid mixture, measured both as the methyl esters (Fig. 1A) and as the benzyl esters (Fig. 1B) is shown in **Table 1.** Variations between the percentage composition of each fatty acid, measured by both methods, averaged less than 1% and varied less than 2% comparing the maximum range of individual determinations. The conversion of the phospholipids to benzyl esters was shown to be complete (>98%) in 30 min by TLC (data not shown). Fig. 1C shows the simultaneous derivatization of a free fatty acid (18:2) as methyl ester and the fatty acids in (16:0)(18:2) phosphatidylcholine as the benzyl esters. **Fig. 2** shows a simultaneous analysis of free fatty acids and phospholipids in bovine retinal rod outer segment (ROS) membranes.

The formation of the benzyl esters from the fatty acid esters in phospholipids was confirmed in each case by GLC–MS (data not shown). Fig. 1B shows that pure phospholipids yield only benzyl esters with the benzyl transesterification reagent (methyl esters appear only to the extent that free fatty acid impurities are present in the phospholipids) if methanol is absent. The benzyl transesterification reagent used to transesterify unknowns (e.g., Fig. 2) should also be used in parallel to transesterify purified pure synthetic control phospholipids (e.g., Fig. 1B), to show that methanol, originally present in the reagent, has been adequately removed by rotary vacuum evaporation.

DISCUSSION

Transesterification of phospholipids or other glycerides is commonly used to prepare fatty acid methyl esters for gas–liquid chromatographic analysis. Boron trifluoride or boron trichloride in methanol are the most frequently used transesterification catalysts (2, 3). In order to drive this reaction to completion, it is necessary to heat the mixture to about 80°C for 10 min, and there are several reports that use of boron trihalide methanol can lead to losses of highly unsaturated fatty acids (11, 12). A more convenient and milder transesterification method was described by McCreary et al. (13) for triglycerides. In this latter method, a 0.2 M solution of a quaternary ammonium base, (m-trifluoromethyl phenyl) trimethyl ammonium hydroxide, was used as a catalyst in methanol and benzene to accomplish quantitative transesterification of triglycerides under mild conditions at room temperature within 30 min (13). No extraction was required after the formation of the fatty acid methyl esters and the samples could be directly injected into the gas chromatograph, which is a further advantage over the boron trihalide methanol procedure. McCreary et al. (13) showed that variations of replicate analysis of pure triglycerides were less than 2%, and they compared this method to a standard sodium methoxide transesterification, which was found to give identical results. We have obtained similar quantitative transesterification of phospholipids as well as oxidized phospholipids in methanol under these mild conditions (10). Any free fatty acids in the samples are quantitatively converted to fatty acid methyl esters in this method by thermal decomposition, in the gas chromatograph, of quaternary ammonium salts formed with the catalyst (5, 6).

TABLE 1. Fatty acid composition of a phospholipid mixture measured as methyl esters and benzyl esters

Fatty Acid	% Methyl Ester[a]	% Benzyl Ester[b]
16:0	31.0 ± 1.3	32.0 ± 1.0
17:0	37.5 ± 1.0	36.5 ± 0.9
18:2	31.5 ± 1.5	31.5 ± 1.6

[a]Four independent samples, typical chromatogram in Fig. 1A.
[b]Eight independent samples, typical chromatogram in Fig. 1B.

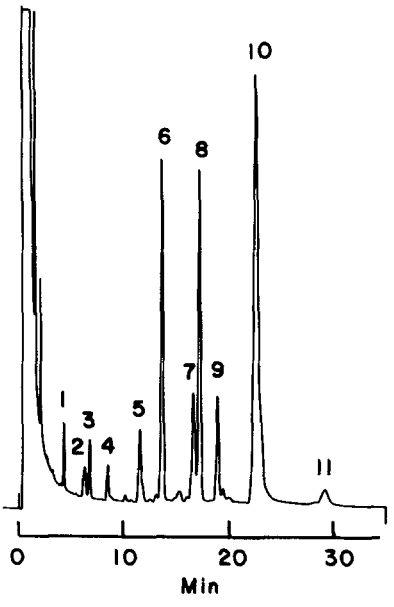

Fig. 2. Analysis of the total fatty acid content of bovine retinal rod outer segment membranes. The methyl esters formed from the free fatty acids are 16:0, 18:1 + 18:2, 18:0, 20:4, and 22:4 + 22:5 + 22:6, and are peaks 1–5, respectively. The benzyl esters formed from the fatty acid esters in phospholipids or glycerides are 16:0, 18:1 + 18:2, 18:0, 20:4, 22:4 + 22:5 + 22:6, 24:4 + 24:5, and are peaks 6–11, respectively. The analysis was performed on a 3% SP 2100 column with temperature programmed from 175 to 250°C, at 4°C/min.

In order to permit simultaneous differentiation of free fatty acids, from the fatty acid esters in phospholipids or other glycerides, we sought to transesterify to a different alcohol than methanol while retaining the ability of determining free fatty acids as methyl esters. We were especially interested in formation of the benzyl esters of the fatty acids because of the possibility of using the benzyl group for absorbance detection in HPLC. Conventional methods of forming benzyl esters require the enzymatic hydrolysis or saponification of phospholipids or other glycerides and subsequent purification of the released fatty acids prior to incubation with benzyl bromide to form the benzyl esters (2, 4).

We were able to modify the method of McCreary et al. (13) to permit the formation of the fatty acid benzyl esters by a one-step transesterification of phospholipids and triglycerides at room temperature. It was found, by replicate transesterification and analysis of phospholipid mixtures, that variations in percentage fatty acid composition were less than 2% (Table 1). The benzyl alcohol is less reactive than methanol; however, the transesterification could be made to go rapidly to completion if the procedure was carried out in the presence of 1% w/v potassium *tert*-butoxide. The method was not tested on mono- or diglycerides, but presumably it will work efficiently in these cases as well.

An important precaution regarding this method is to monitor the completeness of the transesterification by TLC. Potassium *tert*-butoxide is extremely hygroscopic, and may be inactivated by traces of water. This causes a slow, possibly incomplete transesterification reaction, which can be overcome by supplementation with dry potassium *tert*-butoxide. This problem is most serious if humidity is high. The transesterification reagent should be mixed immediately before use.

Contamination of the reaction mixture with methanol or other alcohols should be avoided during and after the transesterification. For example, if methanol is present during the transesterification reaction, a portion of the fatty acid esters in the phospholipids or triglycerides will be transformed into fatty acid methyl esters and part of them will be transformed into benzyl esters. Therefore, it is important to evaporate the dichloromethane phase (collected after total lipid extraction) to complete dryness since there is often methanol present in extracts. In some cases a second dissolution in dichloromethane, followed by drying, may be needed to remove residual methanol. If methanol is added after the phospholipid has been completely transesterified, some of the benzyl esters will be slowly converted to methyl esters if the quaternary ammonium hydroxide catalyst is still present. The catalyst may be removed, if desired, by extracting the derivatives into hexane. After the transesterification reaction, 1 ml of hexane, 200 μl of water, and 200 μl of methanol were added in that order to partition the benzyl esters into the hexane and the catalyst into the methanol-water. No conversion of fatty acid benzyl esters into methyl esters was found by GLC analysis when methanol was added after hexane and water. However, if free fatty acids are to be analyzed, it should be noted that a hexane extraction of the transesterified samples should be avoided because the free fatty acid salts of the quaternary ammonium catalyst may not extract quantitatively into hexane. Tertiary butyl alcohol, formed from reaction of potassium *tert*-butoxide with water in the transesterification reagent, does not form tertiary butyl esters from fatty acid esters in phospholipids, presumably because of steric hindrance.

Biological membranes and other biological samples often contain free fatty acids as well as phospholipids and some other glycerides (14, 15). Typically, fatty acids must first be separated from phospholipids and other glycerides by TLC (e.g., 14) in order to analyze the fatty acid content of membranes or other samples. In the method presented, the fatty acid esters in the phospholipids and triglycerides are converted to benzyl esters, but the free fatty acids are converted upon injection in the gas chromatograph to methyl esters. We performed simultaneous determinations of the free fatty acids as well as the composition of fatty acids esterified in phospholipids or other glycerides in bovine retinal rod outer segment (ROS) membranes. The results, as shown in Fig. 2, agree

with those reported by Drenthe et al. (14) and Miljanich et al. (15) although much more of the most highly unsaturated free fatty acids are recovered, presumably because the TLC step is avoided in the present method. Since there is no detectable 17:0 fatty acid present in ROS membranes (14) (Fig. 2), diheptadecanoyl phosphatidylcholine is a useful internal standard for quantitative analysis.

Fig. 1A–C shows that the fatty acid benzyl esters have much longer GLC retention times than the methyl esters. This is a distinct advantage for simultaneous determination of the fatty acid methyl and benzyl esters since the methyl esters of virtually all of the naturally occurring fatty acids are eluted from the column before any of the benzyl esters of fatty acids commonly found in biological membranes. For example, the peak containing the 22-C acid methyl esters (peak 5 in Fig. 2) elutes well before the palmitic acid benzyl ester (peak 6 in Fig. 2). There are traces of 24- and 26-carbon fatty acids in some membranes and these methyl esters elute more slowly than the benzyl ester of palmitic acid. If fatty acid methyl ester separations are not desired, the retention times of the benzyl esters can be greatly shortened by starting the temperature program at a higher temperature. For example, starting the temperature program at 225°C with the same rate of increase provided a useful pattern of retention times for the fatty acid benzyl esters on the column employed. The fatty acid benzyl esters can be separated by number of double bonds using a more polar liquid phase (data not shown), and increased resolution can also be obtained by using capillary columns instead of packed columns.

If esters of other alcohols are required, the quaternary ammonium salt can be dissolved in the desired alcohol mixed with a suitable aprotic solvent such as methylene chloride. This transesterification method can be used to introduce higher extinction chromogenic alcohols, fluorescent alcohols, or alcohols suitable for GLC with high sensitivity electron capture detection, or negative ion chemical ionization mass spectroscopy. For example, we have used this method of transesterification to form pentafluorobenzyl esters of fatty acid esters and oxidized fatty acid esters in phospholipids, for analysis by GLC-MS using negative ion chemical ionization with detection at the 10-pg level (16).

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