Preparation of FAME from Sterol Esters

Sir:

Glycerolipids, including TAG and phospholipids, are easily converted into FAME by alkali-catalyzed methanolysis for GC analysis of the FA compositions. Although CH_3ONa is most commonly used as an alkali, NaOH- or KOH-catalyzed methanolysis is very convenient and useful (1,2). The procedure is not only simple and rapid but also safe. In contrast, the preparation of FAME from sterol esters is more difficult than that for glycerolipids. For example, FAME can be prepared from many samples of small amounts of blood or breast milk by KOH-catalyzed methanolysis on small pieces of filter paper (3), but the FA composition of sterol esters, which are a major component of blood lipids, cannot be analyzed by this procedure.

In the methanolysis of TAG (2), the reaction proceeds rapidly in a solvent mixture of hexane and 2 M KOH containing methanol. These two solvents are immiscible. TAG and FAME are soluble in hexane but slightly soluble in methanol. The FAME product that has been formed moves to the hexane layer from the methanol layer in which methanolysis is occurring; hence, the reaction is complete within a few minutes at room temperature. Sterol esters are soluble in hexane but almost insoluble in methanol. This is one of the reasons why sterol esters do not react with methanolic KOH under the same conditions as those used for glycerolipids. Fortunately, a few convenient methods have been developed for the preparation of FAME from sterol esters by alkali-catalyzed methanolysis (4,5). These methods, however, utilize harmful solvents such as benzene or diethyl ether. Benzene is a substance that may have effects on the blood-forming organs, liver, and immune system, and it is also carcinogenic to humans. Diethyl ether is very volatile and highly flammable. Ether also may have effects on the central nervous system. These solvents must therefore be treated under a hood. Here, we propose a less harmful solvent system for the preparation of FAME from sterol esters.

The procedure for the preparation of FAME by methanolysis of sterol esters was optimized with cholesteryl oleate. Cholesteryl oleate (1 mg) was dissolved in 0.08 mL methyl propionate in a 2-mL screw-capped glass vial (15 × 32 mm). To this solution was added 0.12 mL 0.84 M NaOH in methanol. Although it is recommended that the methanolic NaOH solution be dried with Molecular Sieves 3A prior to the addition, this treatment is not absolutely essential. The final concentration of NaOH in the total solvent mixture was 0.50 M. After 1 h at 37°C, the reaction was terminated by the addition of 0.01 mL acetic acid. Hexane (0.1 mL) containing methyl heptadecanoate as an internal standard and 0.2 mL H_2O was added to the solution. After vortexing, the hexane layer was injected directly in a packed glass GC column (2 m × 3 mm, 10% SP-2340) or passed through a silica gel cartridge to remove the free cholesterol. In the latter case, FAME were eluted with hexane/methyl acetate (97:3) and the eluent was concentrated for capillary GC (30 m × 0.53 mm, SUPELCOWAX 10; Supelco, Bellefonte, PA).

Methanolysis of sterol esters is expected to proceed in a single solvent phase consisting of sterol esters, an alkali catalyst, methanol, and another solvent that is miscible with methanol. The solvent mixture is required to dissolve both sterol esters and the alkali catalyst. Methyl propionate was selected because it is miscible with methanol, and the mixture of methyl propionate and methanol can dissolve both sterol esters and NaOH or KOH. The optimal ratio of methyl propionate to methanol was 4:6. Methyl propionate gave the best yield of FAME among the solvents tested (benzene, toluene, chloroform, diethyl ether, acetone, methyl acetate, and methyl propionate). Although NaOH was less soluble than KOH in the solvent mixture of methyl propionate and methanol, it was superior to KOH in the yield of FAME (83.5% at 0.5 M of KOH and 87.6% at 0.5 M of NaOH in a preliminary experiment). In the range of concentrations of NaOH higher than 0.6 M, the alkali precipitated during the reaction. At a lower concentration of 0.25 M, the yield was lower by about 5% than at 0.50 M.

With the undried NaOH/methanol, the yields of methyl oleate from cholesteryl oleate were higher than 90%. Under these conditions, a small amount of cholesteryl propionate was formed (Fig. 1, lane 2). Cholesteryl oleate that was adsorbed by the silica gel of the TLC plate was also converted into methyl oleate in somewhat lower yields (87%) than those for neat cholesteryl oleate (Fig. 1, lane 3). Regardless of the presence of silica gel, free oleic acid was a minor product. The release of FFA could be minimized by the use of dried NaOH/methanol. When NaOH is dissolved in methanol, CH_3ONa and H_2O are formed ($CH_3OH + NaOH \rightarrow CH_3ONa + H_2O$). Because alcoholysis is much faster than saponification in alcoholic NaOH solutions, the H_2O that is formed from NaOH and methanol does not affect the yield of FAME in the rapid preparation of FAME from glycerolipids (2). However, methanolysis of sterol esters requires a much longer reaction time; hence, the formation of FFA is inevitable. Because drying the methanolic NaOH solution with Molecular Sieves 3A should result in the preparation of anhydrous methanolic

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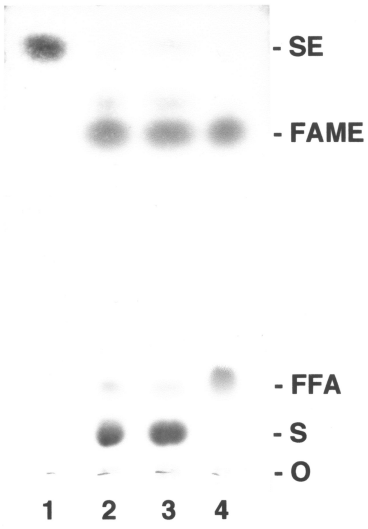

FIG. 1. Identification of reaction products by TLC. Cholesteryl oleate was incubated with a mixture of methyl propionate and 0.84 M NaOH in methanol (2:3) at 37°C for 1 h (lane 2). Cholesteryl oleate was also developed on a silica gel TLC plate in hexane/acetone (96:4). After visualization with 0.01% primuline in methanol, the corresponding band was scraped off into a vial. The cholesteryl oleate was methylated in the presence of silica gel by a similar procedure with minor modifications (lane 3). The pale spot found between SE and FAME in lane 2 or lane 3 is cholesteryl propionate. Lane 1, cholesteryl oleate (before reaction); lane 4, authentic lipids (FAME and FFA). Developing solvent, hexane/*tert*-butyl methyl ether/acetic acid (85:15:0.5). SE, sterol ester (cholesteryl oleate); S, sterol (cholesterol); O, origin.

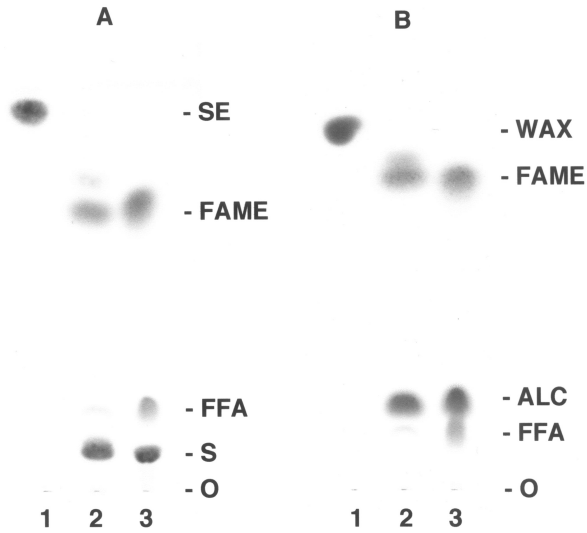

FIG. 2. Methanolysis of cholesteryl oleate and oleyl oleate in the mixture of methyl propionate and a methanolic NaOH solution dried with Molecular Sieves 3A. A methanolic solution of 0.84 M NaOH was dried with Molecular Sieves 3A overnight. (A) Cholesteryl oleate was incubated with methyl propionate and the dried NaOH solution (2:3) at 37°C for 1 h (lane 2). Lane 1, cholesteryl oleate (before reaction); lane 3, authentic lipids (FAME, FFA, and sterol). (B) Oleyl oleate was treated under the same conditions as described above (lane 2). The spot found between WAX and FAME in lane 2 is oleyl propionate. Lane 1, oleyl oleate (before reaction); lane 3, authentic lipids (FAME, FFA, and oleyl alcohol). Developing solvent, hexane/acetone (95:5). WAX, oleyl oleate; ALC, oleyl alcohol; see Figure 1 for other abbreviations.

CH_3ONa, the formation of FFA was negligible with this dried solution (Fig. 2A). The yield of methyl oleate was higher than 94%, indicating that methyl propionate served as a good substitute for benzene or diethyl ether, which had been used earlier (4,5). Wax, i.e., long-chain FA esters of higher alcohols, and sterol esters are also resistant to methanolysis under the conditions for glycerolipids (2). Figure 2B shows that the same procedure as the one proposed for the methanolysis of sterol esters is applicable for wax. The reaction of wax was faster than that of sterol esters and was complete within 40 min. Thus, the combination of methyl propionate and the methanolic NaOH solution dried with Molecular Sieves 3A was an efficient reagent for the methanolysis of sterol esters or wax.

To prevent possible hazards, we would like to recommend the use of this safe solvent system for the preparation of FAME from sterol esters, wax, or lipid samples containing both glycerolipids and sterol esters, such as blood lipids. The procedure utilizes neither benzene nor diethyl ether. Methanol, methyl propionate, and hexane are used as the organic solvents. Methanolic CH_3ONa is commonly prepared from methanol and Na metal, which may cause a fire, whereas the dried methanolic NaOH solution can be more safely and easily prepared than methanolic CH_3ONa.

REFERENCES

1. Glass, R.L., Alcoholysis, Saponification and the Preparation of Fatty Acid Methyl Esters, *Lipids 6*:919–925 (1971).
2. Ichihara, K., A. Shibahara, K. Yamamoto, and T. Nakayama, An Improved Method for Rapid Analysis of the Fatty Acids of Glycerolipids, *Ibid. 31*:535–539 (1996); Erratum, *Ibid. 31*:889 (1996).
3. Ichihara, K., K. Waku, C. Yamaguchi, K. Saito, A. Shibahara, S. Miyatani, and K. Yamamoto, A Convenient Method for Determination of the C_{20-22} PUFA Composition of Glycerolipids in Blood and Breast Milk, *Ibid. 37*:523–526 (2002).
4. Christie, W.W., A Simple Procedure for Rapid Transmethylation of Glycerolipids and Cholesteryl Esters, *J. Lipids Res. 23*:1072–1075 (1982).
5. Zubillaga, M.P., and G. Maerker, Transesterification of Cholesteryl Esters, *J. Am. Oil Chem. Soc. 65*:780–782 (1988).

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