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METHODS

Improved Methods for the Fatty Acid Analysis of Blood Lipid Classes

Ken'ichi Ichihara · Kumiko Yoneda · Ayuko Takahashi · Noriko Hoshino · Motoyoshi Matsuda

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Abstract Two improved methods have been developed for preparation of fatty acid methyl esters (FAME) from major *O*-ester lipid classes in blood, i.e., cholesterol ester, triacylglycerol, and glycerophospholipids. The methods involve simple operations, and use neither harmful solvents such as chloroform or benzene nor highly reactive volatile reagents such as acetyl chloride. The FAME synthesis reaction proceeds under mild temperature conditions. The methods include (1) extraction of lipids from 0.2 ml of blood with 0.2 ml of *tert*-butyl methyl ether and 0.1 ml of methanol, (2) separation of the total lipids into lipid classes using a solid-phase extraction column or thin-layer chromatography, and (3) methanolysis of each lipid class at room temperature or at 45 °C. In all the operations, solvent concentration is performed only once prior to gas–liquid chromatography (GC). No noticeable differences in composition determined by GC have been found between FAME prepared by the present methods and those prepared by a conventional method involving lipid extraction with chloroform/methanol. The mild reaction and simplified procedures of the present methods enabled safe and reproducible analysis of the fatty acid compositions of the major ester-lipid classes in blood.

Keywords Fatty acid composition · Fatty acid methyl ester · Methanolysis · Cholesterol ester · Plasma · Phospholipid · Triacylglycerol · Blood lipids

K. Ichihara (✉) · K. Yoneda · A. Takahashi · N. Hoshino · M. Matsuda
Department of Biomolecular Chemistry, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Shimogamo, Kyoto 606-8522, Japan
e-mail: ichihara@kpu.ac.jp

Abbreviations

tert-BME	*tert*-Butyl methyl ether
CE	Cholesterol ester(s)
FAME	Fatty acid methyl ester(s)
FFA	Free fatty acid(s)
GC	Gas–liquid chromatography
GPL	Glycerophospholipids
GroPCho	Glycerophosphocholine
GroPEtn	Glycerophosphoethanolamine
PtdCho	Phosphatidylcholine
PtdEtn	Phosphatidylethanolamine
SPE	Solid phase extraction
TAG	Triacylglycerol(s)
TLC	Thin-layer chromatography

Introduction

The major ester-lipid classes in blood are cholesterol esters (CE), triacylglycerols (TAG), and glycerophospholipids (GPL). The fatty acid compositions of these lipid classes are among the indicators related to human health and nutritional status, and they have been used in many clinical and epidemiological studies [1–6]. While convenient methods have been presented for preparation of fatty acid methyl esters (FAME) from blood total lipids [7–10], the preparation of FAME from individual lipid classes in blood is both labor-intensive and time-consuming. The first step in conventional preparation of FAME from lipid classes is extraction of total lipids from blood with chloroform–methanol, phase separation, and concentration of the chloroform solution. However, the trihalomethane solvent is toxic for humans and its carcinogenicity is a considerable

health risk for researchers in laboratories [11]. Chloroform may also contaminate the environment. It has been noted that labile lipid species can be chemically modified with the phosgene formed by decomposition of chloroform [12]. Chloroform has another disadvantage in phase separation, in that it forms a lower layer because of its high density (1.48 g/cm^3). When the lower solvent layer is collected with a pipette, it can be contaminated with solid impurities floating in the interface between the upper and lower layers [13]. Therefore, chloroform should be replaced with a less harmful, non-chlorinated, low-density solvent. The second step is isolation of individual lipid classes by silica gel thin-layer chromatography (TLC) or column chromatography. The procedure of this step varies between groups, and there is still no unified protocol for isolation. The third step is formation of FAME from the isolated individual lipid classes by methanolysis or by saponification followed by methylation. Although FAME have been mostly synthesized at temperatures higher than 70 °C, the operation of methanolysis under lower, mild temperatures must be adopted for safety of researchers and to allow simplification of the apparatus used. BF_3 and 3-(trifluoromethyl) phenyltrimethylammonium hydroxide are often used as catalysts for methanolysis of isolated lipid classes [14, 15], but fluorine compounds are restricted for the global environment by local drainage laws. Environmentally friendly alternatives to fluorine compounds should be used as catalysts. HCl is a widely used acid catalyst, and anhydrous HCl/methanol can be prepared from acetyl chloride and methanol [16]. However, the volatile acid halide is an extreme irritant to the eyes, and care must also be taken to prevent violent reaction with methanol. From a safety standpoint, it is desirable to avoid use of this reagent. Benzene, which has been used in the preparation and extraction of FAME [7, 17], may cause leukemia, and it should therefore be replaced by an alternative solvent. Purification of the synthesized FAME is necessary to remove impurities for capillary gas–liquid chromatography (GC) at the last step of preparation, but the procedure is variable and is not standardized. During these operations, concentration of solvents is often repeated, but the number of concentration steps must be minimized for simplification.

We attempted to develop and establish simplified procedures for routine fatty acid analysis of blood CE, TAG, and GPL, and also to minimize possible hazards during chemical manipulation in laboratories and to reduce environmental burden of waste products. Here, we propose two methods and the corresponding protocols for preparation of FAME derived from the blood lipid classes. The first includes a procedure using a solid-phase extraction (SPE) column for separation of blood lipids into lipid classes and is suitable for simultaneous treatment of many samples or robotic systems, and the other includes a TLC procedure, which has been used most frequently to date.

Materials and Methods

Reagents

Cholesteryl oleate and dierucoyl glycerophosphocholine (GroPCho) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesteryl erucate, trierucoyl glycerol, and the FAME standard mixture for GC were purchased from Nu-Chek-Prep (Elysian, MN, USA). Trioleoyl glycerol was obtained from Sigma-Aldrich (St. Louis, MO, USA). Dioleoyl glycerophosphoethanolamine (GroPEtn) was from Wako Pure Chemical Industries (Osaka, Japan). Dioleoyl GroPCho was synthesized from GroPCho and oleic acid [18]. Acetone, chloroform, hexane, and methanol were purchased from Wako Pure Chemical Industries or Nacalai Tesque (Kyoto, Japan) as glass-distilled solvents for analysis of residual pesticides and herbicides. Metal Na, KOH, 50% BF_3 in methanol, *tert*-butyl methyl ether (*tert*-BME), methyl acetate, and heparin sodium salt were of reagent grade. Methanolic CH_3ONa solutions of 1.2 M and 2.0 M were prepared by diluting 25% (w/w; 4.37 M) methanolic CH_3ONa, which was purchased from Sigma-Aldrich, with methanol or prepared by dissolving metal Na in methanol. A reagent for methylation of FFA, 0.8 M HCl in 95% methanol, was prepared by diluting conc. HCl (12 M) 15-fold with methanol. TLC plates of silica gel (0.25 mm thick, No. 105715) and SPE columns packed with 200 mg of silica gel (LiChrolut, No. 102021) were products of Merck (Darmstadt, Germany). The SPE columns were previously washed with 10 ml of hexane and 6 ml of acetone to remove plasticizers and impurities, and then dried in vacuo (1.3 kPa) for 16 h. Microcentrifuge tubes (1.5 ml; Greiner Bio-One, Kremsmünster, Austria) made of homopolymer polypropylene and translucent were soaked in hexane overnight to remove plasticizers. Transparent tubes made of the copolymer polypropylene contained more plasticizers and were not used for lipid extraction. Most solvent systems for lipid extraction and TLC contained 0.001% 2,6-di-*tert*-butyl-*p*-cresol as an antioxidant. Small volumes ($\leq$1 ml) of organic solvents were measured with positive displacement pipettes (Microman M-100 and M-250; Gilson, Middleton, WI, USA). M-1000 was also used for solvents other than chloroform.

TLC and GC

Reaction products of methanolysis were analyzed by silica gel TLC. Developed lipids on TLC plates were visualized

by spraying with 50% (w/w) sulfuric acid and then heating at 137 °C. Phospholipids were also detected with the Dittmer and Lester reagent [19]. For preparation of FAME from lipid classes, methanolic 0.001% primulin was sprayed on plates and lipids were detected under ultraviolet light at 365 nm. FAME prepared were analyzed with a Shimadzu 2014 gas chromatograph equipped with flame ionization detectors and columns of DB-23 (0.25 mm × 30 m) and SUPELCOWAX 10 (0.53 mm × 30 m) at an isothermal column temperature of 200 °C or 215 °C in N_2 gas.

Conventional Method for Preparation of FAME from Blood Lipid Classes

Lipids were extracted from 0.2 ml of blood plasma containing heparin with 4 ml of chloroform/methanol (2:1, v/v) by the Folch method [20]. Total lipids thus obtained were developed on silica gel plates (5 cm in width × 10 cm in height) to 2 cm from the origin with acetone and then redeveloped with hexane/*tert*-BME (90:10, v/v) to 8 cm from the origin. Lipids were detected with primulin, and those located at the origin of the plate were regarded as GPL. Silica gel bands corresponding to lipid classes were scraped off and suspended in 2 ml of methanol/toluene (4:1, v/v). After addition of acetyl chloride (0.2 ml), the mixture was heated at 100 °C for 1 h in a screw-capped glass test tube [7]. FAME formed were extracted with hexane.

Method I (SPE Column Method)

Extraction of Lipids and SPE Column Fractionation (*Fig. 1a*)

Total lipids were extracted from 0.2 ml of whole blood in small glass test tubes or in 1.5-ml polypropylene microcentrifuge tubes by vortexing for 1 min with 0.2 ml of *tert*-BME and 0.1 ml of methanol. The tubes were centrifuged for 1 min and 0.1 ml of the upper ether layer formed was slowly applied at the center of the upper-frit surface of a dry SPE column packed with 200 mg of silica gel. The upper frit put on the silica gel matrix was washed with 0.02 ml of hexane. The silica gel charged with blood total lipids as a *tert*-BME solution containing methanol and water was allowed to stand for 5 min and dried for 1 h or overnight in vacuo (1.3 kPa). CE was eluted with 3.4 ml of 1% (v/v) methyl acetate in hexane, and the volume of eluate was 3.0 ml. TAG was then eluted with 3 ml of 2.5% (v/v) methyl acetate in hexane. After the column was washed with 3 ml of acetone to remove cholesterol and pigments, GPL were eluted with 4 ml of methanol.

(a) Extraction of blood total lipids and isolation of lipid classes by an SPE column

Blood 0.2 ml
- ← 0.2 ml of *tert*-BME and 0.1 ml of methanol
- vortexed for 1 min
- centrifuged for 1 min

tert-BME layer (0.1 ml from 0.14 ml in total)
- applied to a silica gel SPE column

Silica gel SPE column
- washed with 0.02 ml of hexane
- allowed to stand for 5 min
- dried in vacuo for 1 h or overnight
- eluted with 3.4 ml of 1% methyl acetate in hexane (CE)
- eluted with 3 ml of 2.5% methyl acetate in hexane (TAG)
- washed with 3 ml of acetone
- eluted with 4 ml of methanol (GPL)

CE, TAG, and GPL fractions

(b) Preparation of FAME from lipid classes

CE fraction (3 ml of 1% methyl acetate/hexane)
- ← 1.5 ml of acetone and 0.6 ml of 2 M CH_3ONa
- vortexed and allowed to stand for 30 min at room temperature
- ← 0.1 ml of acetic acid and 3 ml of water

Hexane layer
- washed twice with 3 ml each of water
- applied to an SPE column
- eluted with 3 ml of 1% methyl acetate/hexane

Eluate (FAME from CE)
- concentrated

GC

TAG fraction (3 ml of 2.5% methyl acetate/hexane)
- ← 0.1 ml of acetone and 0.1 ml of 2 M CH_3ONa
- vortexed for 30 s at room temperature
- ← 0.02 ml of acetic acid and 3 ml of water

Hexane layer
- washed twice with 3 ml each of water
- applied to an SPE column
- eluted with 3 ml of 1% methyl acetate/hexane

Eluate (FAME from TAG)
- concentrated

GC

GPL fraction (4 ml of methanol)
- ← 0.6 ml of 2 M CH_3ONa
- vortexed and allowed to stand for 7 min at room temperature
- ← 0.1 ml of acetic acid, 3 ml of hexane, and 3 ml of water

Hexane layer
- washed with 3 ml of water
- applied to an SPE column
- eluted with 3 ml of 1% methyl acetate/hexane

Eluate (FAME from GPL)
- concentrated

GC

Fig. 1 Flow chart for Method I

Preparation of FAME from Lipid Classes in Eluates (*Fig. 1b*)

The CE solution (3 ml) was mixed with 1.5 ml of acetone and 0.6 ml of 2 M CH_3ONa, the final concentration of which was 0.24 M. The mixed solution, which formed a single phase, was allowed to stand for 30 min at room temperature. Methanolysis was stopped with 0.1 ml of acetic acid, and then 3 ml of water was added to the solution. After mixing, the hexane layer formed was washed twice with 3 ml of water each time and applied to a silica gel SPE column conditioned with 2 ml of hexane for removal of the byproduct cholesterol and small amounts of FFA. The flow-through fraction was discarded, and FAME were then eluted with 3 ml of 1% methyl acetate in hexane. Cholesterol and FFA were retained in the column. The FAME solution was concentrated in vacuo for GC analysis.

To the TAG solution (3 ml) were added 0.1 ml of acetone and 0.1 ml of 2 M CH_3ONa, and the mixed solution of two separate phases was vortexed for 30 s at room temperature. The reaction was terminated by addition of 0.02 ml of acetic acid, and the solution was mixed with 3 ml of water. The hexane layer was washed twice with water and FAME were purified by the same procedures as described above.

The methanolic GPL solution (4 ml) was mixed with 0.6 ml of 2 M CH_3ONa. Methanolysis was completed within 7 min at room temperature. After addition of 0.1 ml of acetic acid and 3 ml of water to the reaction mixture, FAME were extracted with 3 ml of hexane. The separated hexane layer was washed with 3 ml of water and FAME were purified with a silica gel column to remove non-acylated compounds similar to GPL in polarity as described above.

Method II (TLC Method)

Extraction of Lipids and Fractionation by TLC (*Fig. 2a*)

An area (4 cm in width × 1 cm in height) of a 5 × 10 cm silica gel TLC plate was spotted with 0.1 ml of a *tert*-BME solution of total lipids extracted from 0.2 ml of whole blood by the same procedures as described for lipid extraction in Method I. The plate was dried in vacuo (1.3 kPa) for 15 min, developed with acetone to 3 cm from the bottom of the plate, dried in vacuo for 5 min, and redeveloped with hexane/*tert*-BME (90:10, v/v) to 9 cm from the bottom. Bands of CE and TAG were located by spraying with primulin solution followed by visualizing under an ultraviolet lamp at 365 nm, while GPL remained at the spotted area. Each silica gel band was scraped off and placed in a glass test tube (16.5 mm × 125 mm) for TAG and GPL or in a screw-capped glass test tube (16.5 mm × 105 mm) for CE.

Preparation of FAME from Lipid Classes on Silica Gel (*Fig. 2b*)

CE on silica gel was vortexed with 0.5 ml of toluene/acetone (1:1, v/v) and 1 ml of 1.2 M CH_3ONa for 10 s, and methanolysis was carried out at 45 °C for 30 min. To the reaction mixture was added 3 ml of 0.8 M HCl in 95% methanol, and the test tube was incubated at 45 °C for 20 min for methylation of FFA byproducts. Two phases were formed by the addition of 1 ml of hexane and 2 ml of water to the acidic solution, and the hexane layer was washed with 2 ml of water. The hexane solution thus obtained was applied to a silica gel SPE column. The adsorbed FAME were eluted with 3 ml of 1% methyl acetate in hexane, and the eluate was concentrated for GC analysis.

TAG on silica gel and GPL on silica gel were vortexed with 0.5 ml of toluene/acetone (1:1, v/v) and 1 ml of 1.2 M CH_3ONa for 30 s, and then the mixture was allowed to stand for 1 min at room temperature. Methanolysis was terminated by addition of 0.1 ml of acetic acid. Then, 1 ml of hexane and 2 ml of water were added to the solution for extraction of FAME. The hexane layer was washed with 2 ml of water, and FAME were purified by the same procedure as described for methanolysis of CE.

Assessment of Lipid Extraction with *tert*-BME/Methanol

Whole blood (0.2 ml) was vortexed with 0.2 ml of *tert*-BME and 0.1 ml of methanol, and the mixture was centrifuged. The upper *tert*-BME layer was removed, and the surface of the lower layer was gently washed with 0.1 ml of *tert*-BME. These *tert*-BME solutions were combined. Lipids that were not extracted with *tert*-BME and remained in the lower water/methanol layer were extracted twice with 0.2 ml and with 0.1 ml of chloroform. For comparison, blood (0.2 ml) was diluted with 0.3 ml of 0.5 M KH_2PO_4 and lipids were extracted with 1.5 ml of chloroform and 0.5 ml of methanol by vortexing for 2 min [21]. The chloroform layer was removed, and the surface of the water/methanol layer was gently washed with 1 ml of chloroform. The two chloroform solutions were combined. Lipids in the water/methanol layer were re-extracted twice with 1 ml and with 0.5 ml of chloroform. Extracted lipids were analyzed by TLC. The plate was first developed in chloroform/methanol/water/acetic acid (65:35:4:1, v/v/v/v) to 3 cm from the origin, dried in vacuo, and then redeveloped in hexane/*tert*-BME/acetic acid (85:15:0.5, v/v/v) to 8 cm from the origin.

(a) Extraction of blood total lipids and isolation of lipid classes by TLC

Blood 0.2 ml
- ← 0.2 ml of *tert*-BME and 0.1 ml of methanol
- vortexed for 1 min
- centrifuged for 1 min

tert-BME layer (0.10 ml from 0.14 ml in total)
- applied to a silica gel TLC plate

Lipids on the silica gel plate
- dried in vacuo for 15 min
- developed with acetone to 3 cm from the bottom
- dried in vacuo for 5 min
- developed with hexane/*tert*-BME (9:1, v/v)
- sprayed with 0.001% primulin/methanol
- located under UV light at 365 nm
- scraped off a test tube

CE, TAG, and GPL adsorbed on silica gel

(b) Preparation of FAME from lipid classes

CE on silica gel
- ← 0.5 ml of toluene/acetone (1:1, v/v)
- ← 1 ml of 1.2 M CH_3ONa
- vortexed and then incubated for 30 min at 45°C
- ← 3 ml of 0.8 M HCl/95% methanol
- incubated for 20 min at 45°C
- ← 1 ml of hexane and 2 ml of water
- vortexed and centrifuged

Hexane layer
- washed with 2 ml of water
- applied to a silica gel SPE column

FAME in the silica gel column
- eluted with 3 ml of 1% methyl acetate/hexane

Eluate (FAME from CE)
- concentrated

GC

TAG or **GPL** on silica gel
- ← 0.5 ml of toluene/acetone (1:1, v/v)
- ← 1 ml of 1.2 M CH_3ONa
- vortexed for 30 s
- allowed to stand for 1 min at room temperature
- ← 0.1 ml of acetic acid, 1 ml of hexane, and 2 ml of water
- vortexed and centrifuged

Hexane layer
- washed with 2 ml of water
- applied to a silica gel SPE column

FAME in the silica gel column
- eluted with 3 ml of 1% methyl acetate/hexane

Eluate (FAME from TAG or GPL)
- concentrated

GC

Fig. 2 Flow chart for Method II

Acid-Catalyzed Methanolysis

Although this study focused on the fatty acid analysis of ester lipids, FAME from both *O*-acyl and *N*-acyl lipids were also prepared (Fig. 3). An 8% (w/v) solution of HCl in methanol/water (85:15, v/v) was prepared by diluting 10 ml of conc. HCl with 42.8 ml of methanol [10]. Blood total lipids were extracted with *tert*-BME/methanol and lipid classes were isolated by TLC. Toluene (0.2 ml), methanol (1.5 ml), and the 8% HCl solution (0.3 ml) were added sequentially to each lipid class/silica gel. The final concentration of HCl was 1.2% (w/v), and the solution contained 2.2% (w/v) water derived from conc. HCl. Methanolysis was carried out at 100 °C for 1 h. The reaction mixture was neutralized with 2 ml of 0.5 M $NaHCO_3$, and products were extracted with chloroform for TLC. When FAME were analyzed by GC, they were extracted with hexane without neutralization.

Results and Discussion

Extraction of Total Lipids and Yields of FAME

tert-BME is immiscible with water and its density is lower than water. It has moderate polarity and moderate volatility with boiling point of 55 °C. Its toxicity is lower than chloroform. It is not prone to peroxide formation. It is commercially available as a reagent of high purity in reasonable prices. Thus, *tert*-BME was selected as an alternative solvent to chloroform for lipid extraction. The compositions of blood total lipids extracted by two

Preparation of FAME from lipid classes

CE, TAG, or GPL on silica gel
- ← 0.2 ml of toluene, 1.5 ml of methanol, and 0.3 ml of 8% HCl/85% methanol
- heated at 100°C for 1 h
- ← 1 ml of hexane and 2 ml of water
- vortexed and centrifuged

Hexane layer
- washed with 2 ml of water
- applied to a silica gel SPE column

FAME in the silica gel column
- eluted with 3 ml of 1% methyl acetate/hexane

Eluate (FAME)
- concentrated

GC

Fig. 3 Flow chart for an alternative TLC method utilizing acid-catalyzed methanolysis. See Fig. 2a for extraction of total lipids and isolation of lipid classes

different solvent systems, the present *tert*-BME/methanol system and a modified Folch method that uses chloroform/methanol, were compared by TLC, but no differences in lipid composition were found (Fig. 4). The lower water/methanol phase formed on extraction with *tert*-BME was extracted with chloroform to confirm lipid classes remaining in the lower phase that should be discarded. Small amounts of lipids were distributed to the lower layer, but the lipid compositions of the two layers were very similar to each other on the TLC plate. Matyash et al. [13] also reported that *tert*-BME gave good recoveries for extraction of major lipid classes and was comparable to chloroform used in the Folch method [20] or the Bligh and Dyer method [22].

Extraction efficiencies were compared between the above two solvent systems based on data from an experiment performed in triplicate. An internal standard, methyl erucate, in *tert*-BME or in chloroform was added to blood at the time of lipid extraction. FAME were prepared from the total lipids by HCl-catalyzed methanolysis at 45 °C [10] and analyzed by GC. There was no significant difference in total yield of FAME between *tert*-BME/methanol extraction and chloroform/methanol extraction, and the fatty acid compositions of these FAME preparations were very similar to each other. These findings indicated that *tert*-BME/methanol compares favorably with chloroform/methanol for extraction of blood total lipids as reported previously in lipidomics research [13].

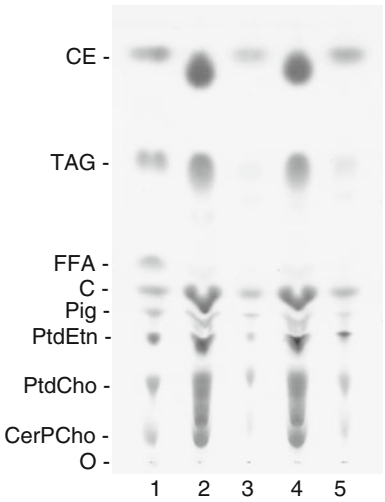

Fig. 4 Lipid extraction from blood with *tert*-BME/methanol and with chloroform/methanol. The TLC plate was first developed in chloroform/methanol/water/acetic acid (65:35:4:1, v/v/v/v) to 3 cm from the origin, dried in vacuo, and then redeveloped in hexane/*tert*-BME/acetic acid (85:15:0.5, v/v/v). *Lane 1* authentic lipids, *lane 2* lipids extracted with *tert*-BME, *lane 3* lipids in the water/methanol layer of *tert*-BME extraction, *lane 4* lipids extracted with chloroform, *lane 5* lipids in the water/methanol layer of chloroform extraction. *C* cholesterol, *Pig* pigments, *CerPCho* sphingomyelin, *O* origin

Methanolysis of Lipid Classes Separated by Silica Gel SPE Columns (Method I)

In the 1980s, SPE columns were used for separation between non-polar lipids and phospholipids [23]. Figure 5a shows that CE, TAG, and GPL could be fractionated from total lipids by SPE columns. Methanolysis was carried out at room temperature without concentration of the lipid solutions, and FAME derived from each lipid class were purified to a single spot on TLC (Fig. 5b).

Methanolysis of TAG proceeds in a solvent mixture of hexane and methanol containing a base catalyst, and it requires vortexing for 2 min at room temperature in the absence of acetone [24]. Addition of acetone to a mixture of the TAG eluate and CH_3ONa/methanol stimulated the reaction, and TAG was converted into FAME within 30 s by vortexing at room temperature (Fig. 5b, lanes 5, 6). Hexane and methanol are immiscible, while methanolysis occurs in the lower methanol phase in which a base catalyst, e.g., CH_3ONa or KOH, is dissolved. As TAG, which is a non-polar lipid, is soluble in hexane but slightly soluble in methanol, it is distributed unevenly to the hexane phase when dissolved in a mixed solvent of hexane and methanol. The concentration of TAG in the methanol phase is low, and hence methanolysis proceeds slowly. When a small volume of acetone is added to a mixture of hexane and methanol, acetone is miscible with methanol and the two polar solvents form a lower phase. As acetone is a good solvent for TAG, the concentration of TAG increases in the lower phase where methoxide ions are present and methanolysis occurs. FAME formed are more hydrophobic than TAG and are transferred immediately to the upper hexane phase. FAME are, therefore, not hydrolyzed by alkali dissolved in the methanol/acetone phase. This is probably why methanolysis of TAG is stimulated with acetone.

CE is more hydrophobic and less soluble in methanol than TAG, and methanolysis of CE does not proceed under the conditions for TAG [25, 26]. Various solvents have been used to increase the solubility of CE in methanol to enhance its reactivity. For example, good yields of FAME are obtained in 1 h at 37 °C or at room temperature by reaction in diethyl ether/methyl acetate/methanol containing 1 M CH_3ONa (50:1:1, v/v/v) or in methyl propionate/methanol containing 0.84 M NaOH (2:3, v/v), respectively [25, 26]. A mixture of CE eluate (3 ml of 1% methyl acetate in hexane), 1.5 ml of acetone, and 0.6 ml of 2 M CH_3ONa/methanol formed a single homogenous phase of the solvents, and methanolysis was completed in 30 min at room temperature (Fig. 5b, lane 2). This improved reactivity of CE was also due to its high solubility in the solution containing acetone.

To a methanol eluate (4 ml) of GPL was added 0.6 ml of 2 M CH_3ONa/methanol to form a 0.26 M CH_3ONa

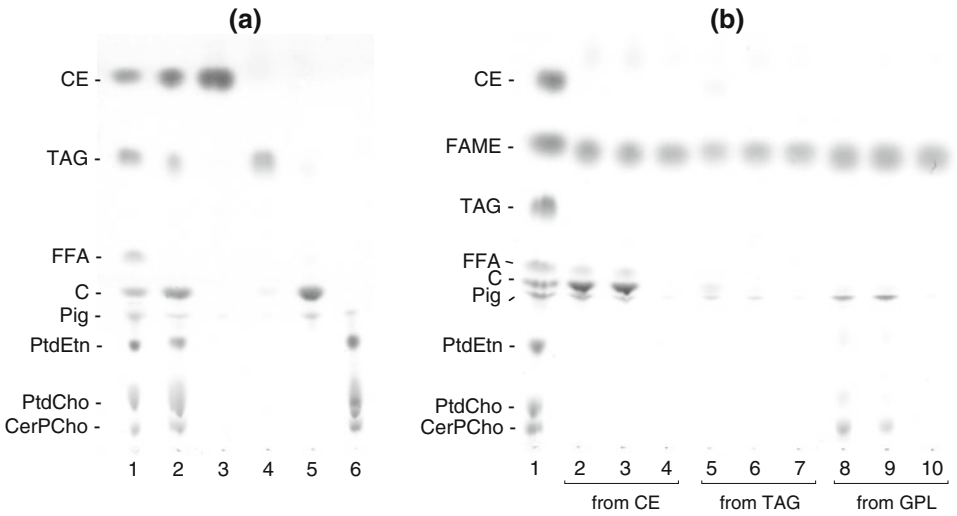

Fig. 5 **a** Isolation of CE, TAG, and GPL by a silica gel SPE column. Total lipids were extracted from whole blood with *tert*-BME/methanol (*lane 2*) and separated into three lipid classes, CE (*lane 3*), TAG (*lane 4*), and GPL (*lane 6*) by an SPE column. *Lane 5* is the acetone eluate that contains cholesterol and pigments. Reference lipids were developed in lane 1. **b** Preparation of FAME from lipid classes. Reaction products of the isolated lipid classes by methanolysis were extracted with chloroform (*lanes 2, 5, 8*) or with hexane (*lanes 3, 6, 9*). The hexane extracts were purified by SPE columns (*lanes 4, 7, 10*). Lane 1 is a mixture of authentic lipids. The conditions of TLC were the same as those in Fig. 4

solution, which was allowed to stand for 7 min at room temperature. As GPL are polar lipids and sufficiently soluble in methanol, the reaction proceeded rapidly in methanol (Fig. 5b, lane 8). The reaction time of 7 min at room temperature was sufficient for transesterification of GPL bearing *O*-ester linkages. The pale spot detected near the position corresponding to PtdEtn after methanolysis was not a phospholipid, and another pale spot found near the position of PtdCho was lysoplasmalogen. Sphingomyelin, which has no ester-linked acyl residue, remained unchanged as expected. The fatty acid composition of sphingomyelin is considerably different from those of glycerophospholipids, and it must be determined using acid-catalyzed methanolysis after isolation.

Acetone is known to reacts with aminophospholipids to form Schiff's bases under mild conditions [27, 28], but the influences of acetone complexes on the preparation of phospholipids and analysis of fatty acid composition were not considered in the present study.

Methanolysis of Lipid Classes Separated by Silica Gel TLC (Method II)

CE, TAG, and GPL were isolated by silica gel TLC from *tert*-BME extracts (Fig. 6). Methanolysis in the presence of silica gel has some disadvantages compared to the reaction in the absence of silica gel, especially for CE; i.e., the reaction proceeds slowly and FFA are produced. However, these disadvantages could be alleviated by optimizing reaction conditions. A reaction medium consisting of 0.25 ml of toluene, 0.25 ml of acetone, and 1 ml of methanolic 1.2 M CH_3ONa forms a single organic phase, in which the concentration of CH_3ONa is 0.8 M. This solvent system containing the base catalyst was able to dissolve all three lipid classes and was used for methanolysis.

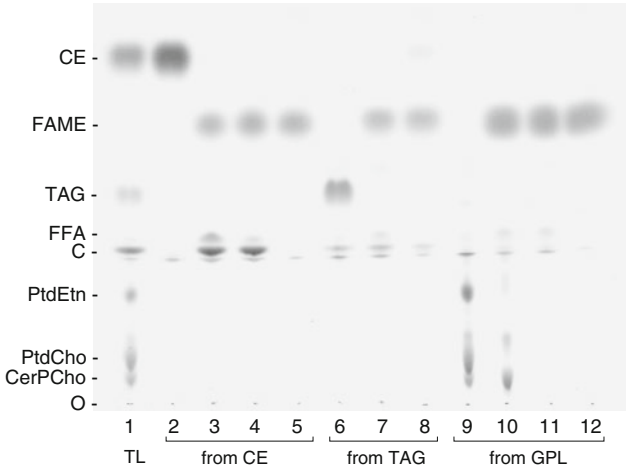

Fig. 6 Preparation of FAME from lipid classes isolated by TLC. *Lane 1* total lipids extracted with *tert*-BME/methanol, *lane 2* CE isolated by TLC, *lane 3* methanolysis products of CE/silica gel, *lane 4* products from CE on silica gel by methanolysis followed by methylation (methanolysis/methylation), *lane 5* FAME purified from methanolysis/methylation products corresponding to lane 4, *lane 6* TAG isolated by TLC, *lane 7* methanolysis products of TAG/silica gel, *lane 8* FAME purified from methanolysis products corresponding to lane 7, *lane 9* GPL isolated by TLC, *lane 10* chloroform extract of methanolysis products from GPL/silica gel, *lane 11* hexane extract of methanolysis products from GPL/silica gel, *lane 12* FAME purified from the hexane extract corresponding to lane 11. The conditions of TLC were the same as those in Fig. 4. *TL* total lipids

Table 1 Blood fatty acid compositions (%) determined by different methods

Lipid class	Fatty acid	Method I	Method II	Conventional method
CE	14:0	1.1 ± 0.2	0.9 ± 0.0	0.8 ± 0.0
	16:0	12.4 ± 0.1[a]	13.8 ± 0.4	13.7 ± 0.0[a]
	16:1	2.8 ± 0.1	2.5 ± 0.1	2.8 ± 0.0
	18:0	1.4 ± 0.1	1.0 ± 0.1	1.0 ± 0.0
	18:1	20.1 ± 0.1	19.9 ± 0.1	20.4 ± 0.0
	18:2	45.6 ± 0.1	45.6 ± 0.3	45.7 ± 0.1
	18:3 n-3	0.8 ± 0.0	0.8 ± 0.0	0.7 ± 0.0
	20:3 n-6	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.0
	20:4 n-6	5.2 ± 0.1	5.2 ± 0.1	5.0 ± 0.0
	20:5 n-3	8.3 ± 0.1	7.9 ± 0.1	7.7 ± 0.0
	22:6 n-3	2.1 ± 0.1	2.0 ± 0.0	1.9 ± 0.0
TAG	14:0	3.0 ± 0.3	2.5 ± 0.1	3.3 ± 0.2
	16:0	25.7 ± 0.2	26.5 ± 0.2	27.2 ± 0.1
	16:1	3.9 ± 0.1	4.1 ± 0.0	4.2 ± 0.1
	18:0	6.6 ± 0.1[a,b]	5.8 ± 0.0[a]	5.7 ± 0.0[b]
	18:1	36.2 ± 0.1	35.5 ± 0.1	35.3 ± 0.1
	18:2	12.2 ± 0.1	12.6 ± 0.0[a]	12.1 ± 0.0[a]
	18:3 n-3	1.1 ± 0.1	1.3 ± 0.0[a]	1.0 ± 0.0[a]
	20:1	2.0 ± 0.1	1.6 ± 0.0	1.5 ± 0.0
	20:4 n-6	1.2 ± 0.0	1.3 ± 0.1	1.2 ± 0.0
	20:5 n-3	2.3 ± 0.0	2.5 ± 0.0	2.5 ± 0.0
	22:5 n-3	1.4 ± 0.0	1.3 ± 0.0	1.2 ± 0.0
	22:6 n-3	4.6 ± 0.1	5.0 ± 0.1	4.7 ± 0.0
GPL	14:0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
	16:0	24.6 ± 0.2	24.8 ± 0.3	24.1 ± 0.2
	16:1	0.4 ± 0.0	0.3 ± 0.0	0.5 ± 0.0
	18:0	15.8 ± 0.1	15.6 ± 0.2	16.4 ± 0.2
	18:1	14.2 ± 0.1	14.2 ± 0.1	14.2 ± 0.0
	18:2	13.0 ± 0.0[a]	12.9 ± 0.1[b]	11.6 ± 0.0[a,b]
	20:1	0.4 ± 0.0[a]	0.3 ± 0.0[b]	0.7 ± 0.0[a,b]
	20:3 n-6	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0
	20:4 n-6	9.0 ± 0.0[a]	9.0 ± 0.1[b]	10.0 ± 0.0[a,b]
	20:5 n-3	6.6 ± 0.0	6.6 ± 0.0	6.5 ± 0.1
	22:4 n-6	0.6 ± 0.0[a]	0.6 ± 0.0	0.8 ± 0.0[a]
	22:5 n-3	2.4 ± 0.0[a]	2.5 ± 0.0	2.8 ± 0.0[a]
	22:6 n-3	11.8 ± 0.1	11.7 ± 0.1	11.3 ± 0.1

Each value is the average obtained from three FAME preparations. Values that share common superscript letters in each row are significantly different at $P < 0.01$. Minor components and C_{24} acids are omitted

In the absence of silica gel, TAG can be readily derivatized to FAME in two solvent phases composed of hexane and methanol [24]. Acetone in the methanol phase enhances the reaction rate, as described for methanolysis of TAG isolated by SPE columns. Silica gel interfered with the reaction, and under the conditions used for the two-phase solvent system, conversion of TAG to FAME resulted in low yields. However, methanolysis of TAG was completed in 90 s at room temperature with trace amounts of FFA byproducts, when silica gel that adsorbed TAG was treated with the homogeneous solution of 0.8 M CH_3ONa in toluene/acetone/methanol (Fig. 6, lanes 6–8).

CE on silica gel was also incubated with CH_3ONa solution (Fig. 6, lanes 2–5). CE disappeared within 30 min at 45 °C or 45 min at 37 °C, but considerable amounts of FFA were produced, as reported previously [25]. The compositions of the FFA were not different from those of FAME formed by methanolysis. FFA in the reaction mixture were scavenged by adding 3 ml of 0.8 M HCl in 95% methanol. Most of the FFA were methylated at 45 °C for

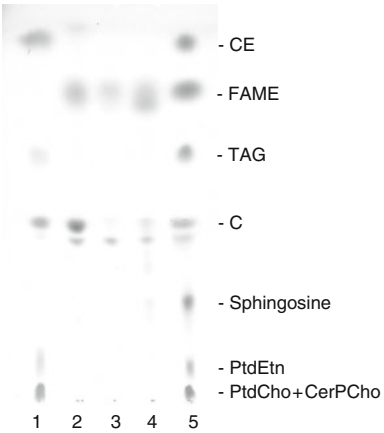

Fig. 7 Methanolysis of blood lipid classes with 1.2% HCl/methanol/toluene in the presence of silica gel. See "Materials and Methods" for details on preparation of FAME. The TLC plate was developed to 3.5 cm from the origin in chloroform/methanol/15 M NH_3 (80:20:1, v/v/v), and then redeveloped in hexane/*tert*-BME (90:10, v/v). *Lane 1* blood total lipids; *lanes 2–4* products of CE, TAG, and phospholipids, respectively; *lane 5* authentic lipids

20 min. Remaining FFA and cholesterol derived from CE were removed by SPE columns prior to GC.

FAME were formed from GPL within 90 s at room temperature by the same procedures as described for TAG, but they were contaminated with sphingomyelin, lysoplasmalogen, pigments, and other impurities (Fig. 6, lanes 9–12). Sphingomyelin and lysoplasmalogen in the reaction mixture showed little extraction by hexane. Pigments and other impurities were derived from the original GPL preparation and were removed by SPE columns.

Purification of FAME by Silica gel SPE Columns

Methanolysis of CE inevitably produces free cholesterol as a byproduct. As methanolysis of acyl lipids is accompanied by a side reaction, saponification or hydrolysis, in the presence of water, small amounts of FFA are formed during the reaction. FAME products obtained under the present conditions of methanolysis were contaminated by cholesterol, FFA, and other impurities, such as pigments. These impurities were removed by SPE columns (Figs. 5, 6). Purification of FAME is thus recommended, but FAME products can be injected immediately into GC columns without purification to speed up analysis as adopted in most methods reported previously [1–3, 7]. Reactions of blood lipids in HCl/methanol at high temperatures are accompanied by the formation of two major artifacts derived from CE and cholesterol [29, 30], i.e., 3,5-cholestadiene and cholesteryl methyl ether, the latter of which cannot be removed by silica columns. Base-catalyzed methanolysis used in the present method does not generate these artifacts.

Fatty Acid Compositions of Lipid Classes and Yields of FAME

The three major *O*-acyl lipids of blood, CE, TAG, and GPL, were isolated by silica gel SPE columns or TLC, and converted into FAME. The fatty acid compositions of these lipid classes were determined by GC and they were compared with each other and with those obtained by a conventional method (Table 1). There were no remarkable differences in fatty acid composition among these methods, although some small but significant differences were found. To assess the validity of the present methods, the yields of FAME were compared between the present methods and a conventional method including Folch extraction followed by isolation of lipid classes by TLC. Total lipids were extracted with the *tert*-BME or chloroform solvents containing three internal standards, i.e., cholesteryl erucate, glyceryl trierucate, and dierucoyl GroPCho. In GC analysis of the FAME thus prepared, similar yields were obtained for the two present methods and the conventional method (data not shown). These findings support the validity and reliability of the present methods. Although fatty acid analysis of lipid classes in whole blood was reported here, plasma and serum can also be analyzed.

Some fatty acid species are acid- and heat-labile. For example, conjugated linoleic acids that occur predominantly in dairy products and show physiological activities are associated human plasma lipids in small quantities [31]. As these acids are unstable under acidic conditions, Methods I and II, which include base-catalyzed methanolysis under mild temperature conditions, will be useful for determination of these acid-labile fatty acid species in plasma lipid classes.

When the combined fatty acid compositions of both *O*-acyl and *N*-acyl phospholipids including sphingomyelin are required, acid-catalyzed methanolysis must be used. Even in this case, a combination of lipid extraction from blood with *tert*-BME/methanol and methanolysis at 100 °C with 1.2% HCl/methanol prepared from aqueous conc. HCl and methanol [10] is recommended for safety and convenience (Fig. 3). Figure 7 shows methanolysis products obtained by this combination. A pale spot found near the solvent front in lane 2 is probably 3,5-cholestadiene, a decomposition product of cholesterol.

In conclusion, the present protocols for preparation of FAME from lipid classes of blood do not include chloroform in the lipid extraction and TLC steps. The reactions of all three lipid classes, CE, TAG, and GPL, proceed at room temperature or at 45 °C. Method I in which lipid classes isolated by SPE columns may be more readily automated than Method II in which silica gel bands corresponding to individual lipid classes on TLC plates must be manually

scraped off. TLC, on the other hand, has been commonly used for separation of blood total lipids into lipid classes. In each method, the protocols proposed are thus safer for researchers and have less impact on the environment than those adopted previously. Moreover, the reaction conditions are mild for lipids. In the future, it may be worthwhile to further develop these methods to simpler and time-saving direction.

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