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Short communication

Purification of fatty acid methyl esters by high-performance liquid chromatography

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Abstract

The determination by gas chromatography (GC) of fatty acid methyl esters (FAMEs) prepared from complex biological samples is subject to interference from cholesterol. During sample injection on the GC system of FAMEs prepared from tissues that contain cholesterol, we observed a major contaminant that co-eluted with docosahexaenoic acid (DHA, 22:6*n*-3). To address this problem, FAMEs were purified on an amino-phase high-performance liquid chromatography (HPLC) column using a hexane–isopropanol gradient. The HPLC retention times for both the FAME fraction and cholesterol were stable and reproducible when the amino column was used for sample purification. The purified extracts were analyzed by GC without artifacts or impurity peaks after 50 analytical runs. The method described here will be useful for measurement of 22:6*n*-3 and other fatty acids important for studies of nutrition or pathology.

Keywords: Fatty acid methyl esters; Cholesterol

1. Introduction

The determination of the fatty acid profile of biological samples is generally accomplished through preparation of fatty acid methyl esters (FAMEs), followed by gas chromatography (GC) analysis. This is a straightforward procedure for samples that contain only phospholipids or triglycerides. However, co-injection of cholesterol along with the FAMEs can lead to a complex mixture of late-eluting peaks on the GC trace, which interfere with the analysis of docosahexaenoic acid (DHA, 22:6*n*-3), 20:5*n*-3 and other highly-retained fatty acids.

The nutritional importance of diets rich in *n*-3 fatty acids has received increasing emphasis. For example, *n*-3 fatty acids have special importance in preventing sudden death from cardiac arrhythmias [1], and diets rich in these fatty acids may prevent or ameliorate atherosclerosis [2], arthritis [3], and depression [4]. The primate requirement of *n*-3 fatty acids, which are necessary for retina and brain function [5,6], can be evaluated by GC measurement of 22:6*n*-3 in samples of nervous tissue [7]. Since plasma and nervous tissue contain abundant cholesterol, preparation of FAMEs from these samples may lead to interference from the cholesterol artifact.

For the abundant fatty acids, including 16:0, 18:0, 18:1*n*-9, 18:2*n*-6 and 20:4*n*-6, standard GC methods are fully adequate, but for fatty acids present at 1% or less of total fatty acids, or for late-eluting fatty acids where cholesterol can interfere, alternative methods may be needed. We have established an

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high-performance liquid chromatography (HPLC) procedure, using an amino-bonded phase column for sample purification, to eliminate free cholesterol from the FAME sample.

2. Materials and methods

2.1. Reagents and apparatus

Acetyl chloride, for preparation of FAMEs, was obtained from Sigma (St. Louis, MO, USA). Hexane, methanol, isopropanol, dichloromethane and benzene were all HPLC-grade. FAME standards, 3,5-cholestadiene, cholesterol and butylated-hydroxy-toluene (BHT) were obtained from Sigma. Chloroform–methanol (2:1) used for the Folch extraction contained 50 μg/ml BHT. The FAMEs were purified by HPLC on an HP-1100 gradient HPLC system (Hewlett-Packard, Avondale, PA, USA), using a Waters amino bonded-phase column (MicroBondapak, 10 μm particle size, 30 cm×3.9 mm I.D.) (Waters, Milford, MA, USA). Amino bonded phase HPLC columns from other vendors gave comparable results. FAME analysis was carried out using an HP-5890 capillary gas chromatograph equipped with a flame ionization detection (FID) system (Hewlett-Packard).

2.2. Plasma lipid extraction and preparation of FAMEs

Total lipids were isolated from 50 μl plasma using a Folch extraction [8]. The plasma lipid residue was dissolved in 0.25 ml benzene, and the fatty acids were methylated by addition of 0.75 ml of a freshly-prepared solution of 10% (w/v) acetyl chloride in methanol [9], followed by heating at 70°C for 2 h.

To extract the FAME fraction from the reaction mixture, the sample was allowed to cool, and 1.2 ml of 7% aqueous NaCl was added, followed by 2 ml hexane. The samples were then vortex-mixed for 30 s, centrifuged for 2 min at 2000 *g*, and the FAME-containing upper hexane layer collected. To make a concentrated sample for injection on the HPLC system, the hexane was evaporated under nitrogen, and the sample re-dissolved in 100 μl hexane.

2.3. FAME purification by HPLC

HPLC purification was carried out with an 8 min linear gradient, from 2% isopropanol to 10% isopropanol, with 1 ml/min flow-rate. The crude FAME extract, containing cholesterol, was injected in a 100 μl volume of hexane onto the HPLC column. Use of hexane as the HPLC injection solvent is optimal to maintain clear separation between FAMEs and cholesterol.

The FAMEs from biological samples all eluted within a narrow window during HPLC purification. To establish that elution window, the retention profile of a mixed FAME standard (Supelco) was monitored at 220 nm on the HPLC system. Once determined with a standard FAME mixture, this collection interval is employed for isolation of FAMEs prepared from plasma samples.

The FAME sample used to determine the collection interval during HPLC purification should not contain BHT, as it will generate an additional peak at 220 nm. After the collection interval is established, the presence of BHT in the sample does not pose difficulties. The cholesterol elution time during the HPLC step was also monitored by absorbance at 220 nm.

The FAME-containing fraction, which eluted between 2.5 and 4 min on the Waters amino column, was collected in a 4-ml glass test tube containing 10 μl of a 2 μg/ml solution of BHT in dichloromethane as an antioxidant. The collected eluent was subsequently evaporated under nitrogen at ambient temperature and the residue resuspended in 100 μl dichloromethane. Sample FAME aliquots in dichloromethane were stored at −20°C for GC analysis.

Following each purification run, the HPLC column was flushed for 2 min with 50% isopropanol at 2 ml/min, and re-equilibrated with hexane–isopropanol (98:2) at 2 ml/min for 4 min.

2.4. FAME analysis by GC–FID

The majority of GC analyses were carried out with an Alltech AT-WAX column (30 m×0.32 mm I.D., 0.50 μm film thickness). For some analyses we employed a Supelco Supelcowax column (30 m×0.32 mm I.D., 0.25 μm film thickness). The

initial column temperature was 100°C, with a ramp at 8°C/min to 250°C. The column temperature was maintained at 250°C for an additional 8 min to elute long chain polyunsaturated fatty acids (PUFAs). Column head pressure was 15 p.s.i. (1 p.s.i.= 6894.76 Pa). Splitless injection, with split on at 30 s, was used to maximize sensitivity for trace FAME components. Dichloromethane was chosen as a GC injection solvent over hexane due to superior peak symmetry and chromatographic properties.

3. Results

3.1. GC of non-purified FAME samples

Consecutive GC analyses of FAME samples prepared directly from plasma demonstrated the gradual appearance of a late-eluting major contaminant. This peak was not observed during the initial GC analysis (Fig. 1, upper trace), but began to appear after several chromatograms (Fig. 1, middle trace). After 10 sample analyses, the contaminant peak was very large (Fig. 1, lower trace) and its total area was comparable to all the FAMEs in the sample. On GC columns we have examined, this contaminant co-elutes in the region of 22:6*n*-3, which renders the measurement of this compound difficult or impossible at low levels of 22:6*n*-3 (ca. 1% of total plasma FAMEs). This contaminant peak was also observed after repeated injections of cholesterol (10 μg/analysis).

3.2. HPLC purification of total plasma FAMEs, with removal of cholesterol

By use of HPLC, with amino-bonded phase and hexane–isopropanol gradient, both FAMEs and cholesterol can be completely separated (Fig. 2). We injected 200 μg of a FAME mixture, composed of different saturated and unsaturated FAMEs. This entire FAME mixture elutes as a broad peak between 3 and 4 min on the Waters amino Microbondapak HPLC column (Fig. 2). Free cholesterol (50 μg) elutes on this HPLC column between 6 and 7 min (Fig. 2).

To verify the elution profile for FAMEs, samples of HPLC eluent were collected every 0.5 min, and each column fraction was analyzed for FAMEs by GC. By this method, all of the FAMEs eluted off the HPLC column between 3 and 4 min. HPLC fractions were analyzed for cholesterol with an enzymatic

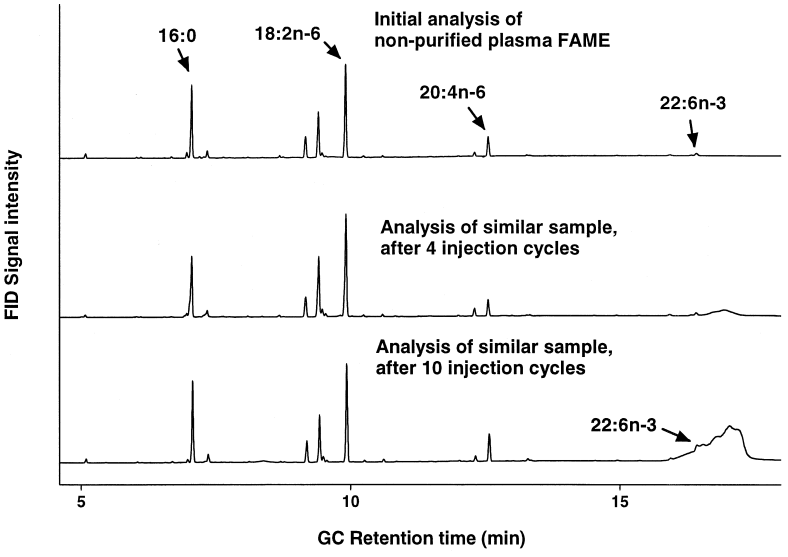

Fig. 1. Effect of repeat GC analysis of non-purified plasma FAME samples. Upper trace: initial injection. Middle trace: after four injection cycles. Lower trace: after 10 injection cycles. Column: Supelcowax, 30 m×0.32 mm I.D., 0.25 μm film thickness. He carrier gas, 15 p.s.i. column head pressure. Initial column temperature, 100°C, with ramp at 8°C/min to 250°C. Injection port: split off, then 20/1 split after 30 s.

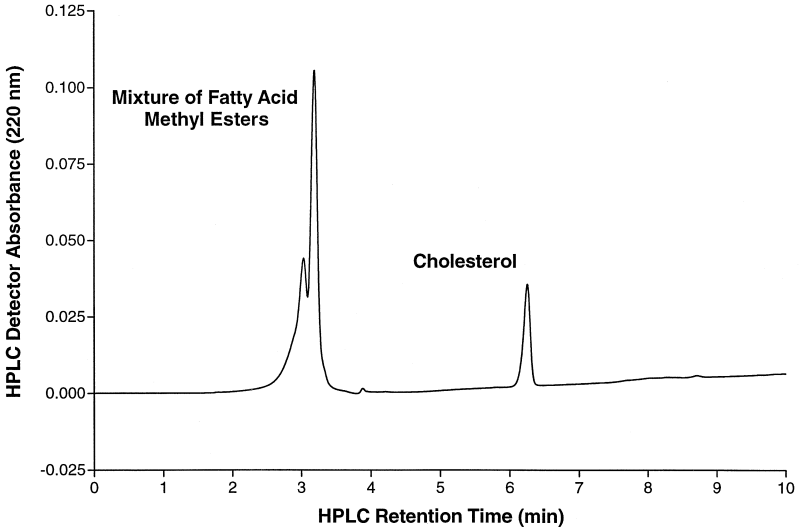

Fig. 2. HPLC analysis of a mixture of FAME standards and cholesterol standard. HPLC column: Waters Microbondak Amino, 10 μm particle size, 30 cm×0.46 cm I.D. Initial mobile phase: hexane–isopropanol (98:2), with 8 min linear ramp to 10% isopropanol. Mobile phase flow-rate=1 ml/min. This retention time profile is stable with the amino HPLC column over the course of numerous sample purifications.

method [10], which confirmed that cholesterol eluted from the HPLC column between 6 and 7 min.

The HPLC retention times established for FAMEs and cholesterol were reproducible over 200 sample purifications using the amino column, so that the FAME fraction could be reliably isolated by collecting the HPLC eluent over the interval from 2.5 to 4 min.

3.3. GC of purified FAME samples

Following HPLC purification, plasma FAME samples generate a GC chromatogram that remains free the late-eluting artifact peak after many analyses. Fig. 3 illustrates the initial analysis of purified plasma FAMEs (upper trace), and a GC analysis following 20 sample runs (lower trace).

3.4. Fatty acid profile of FAME mixture after HPLC purification

The purified FAME mixture showed the same GC profile as the starting material. We analyzed a mixture of nine FAMEs (16:0, 18:0, 18:1*n*-9, 18:2*n*-6, 18:3*n*-6, 18:3*n*-3, 20:4*n*-6, 20:5*n*-3, and 22:6*n*-3), before and after HPLC purification, and obtained identical results on the GC with each sample. This result documented that the FAME profile was not affected by the HPLC purification step.

3.5. Evaluation of amino-bonded phase cartridges

We also attempted to purify samples with amino-bonded phase cartridges (Waters, Sep-Pak), and employed HPLC to determine if cholesterol was completely removed. However, traces of cholesterol were still present after the use of the amino cartridge, which led to the cholesterol artifact after repeat injections on the GC system. Since the presence of residual cholesterol will still yield a substantial artifact upon repeated FAME injections, the complete removal of cholesterol is optimal for precise measurement of 22:6*n*3 and other late-eluting FAMEs.

3.6. GC column stability and FAME purity

When non-purified FAME samples containing cholesterol were injected onto the GC column, periodic prolonged heating of the column (250°C, for 12 h) was needed to remove baseline contaminants, which led to deterioration of the GC column. By

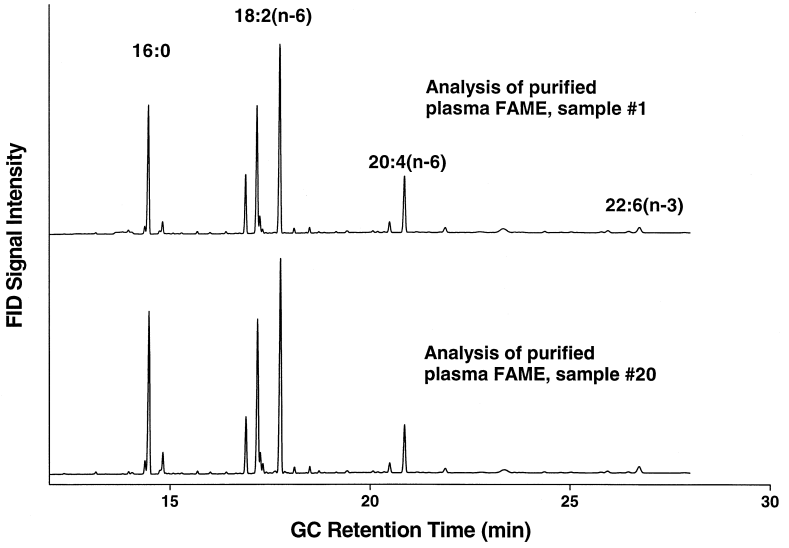

Fig. 3. Repeat GC analysis of plasma FAMEs, after HPLC purification to remove free cholesterol. Upper trace: initial analysis. Lower trace: after 20 injection cycles. Column: Alltech AT-WAX, 30 m×0.32 mm I.D., 0.50 μm film thickness. He carrier gas, 15 p.s.i. column head pressure. Initial column temperature, 100°C, with ramp at 8°C/min to 250°C. Injection port: split off, then 20/1 split after 30 s.

contrast, long GC column lifetime was obtained after injection of HPLC-purified FAME samples, since the purified samples were free of cholesterol.

4. Discussion

We have described the use of HPLC, with amino-bonded phase, to prepare purified FAMEs (Fig. 2). This procedure yield samples free of cholesterol, which allows multiple FAME analyses by GC without artifact formation (Fig. 3). The amino-bonded phase provides separations similar to silica, but retention times for FAMEs and cholesterol demonstrate excellent long-term reproducibility. Use of unbonded silica for the HPLC stationary phase may lead to long-term changes in retention time, due to variation in the amount of water associated with the silica packing. However, this problem is avoided by the use of the amino-bonded phase HPLC column for purification of FAME.

This protocol is not suitable for measurement of hydroxy-fatty acids, which are retained along with cholesterol during sample purification on the HPLC amino-phase column. The method described here may also remove other polar compounds from the initial FAME sample, such as free fatty acids, plant sterols and nitrogenous bases. Some cholestadiene is generated during transesterification with the acetyl-chloride/methanol reagent (broad peak at 23 min in Fig. 3), as is typically found with acid-catalyzed transesterification [11,12], and cholestadiene co-purifies with the FAME fraction on HPLC. Cholestadiene may co-elute with some fatty acid components, which were not identified; however, cholestadiene did not interfere with the analysis of 20:5*n*-3 and 22:6*n*-3.

Injection onto the GC column of underivatized cholesterol can result in the formation of cholesterol dehydration products in the injection port [13] and cholesterol can react with carboxylic acids found in the injection solvent [14]. The polar character of GC columns used for FAME analysis may enhance the tendency of free cholesterol to adhere to the stationary phase and form degradation products. If many samples are to be analyzed for 22:6*n*-3, this HPLC purification procedure may be utilized to prevent the late-eluting cholesterol artifact (Fig. 1). The removal of cholesterol prior to GC analysis has other benefits as well, since this step prevents the formation of cholesterol degradation products that appear throughout the chromatogram. Column lifetime is enhanced

if cholesterol is removed prior to GC injection. The measurement of trace fatty acid components, such as *cis*-isomers and other regioisomers, is enhanced with the highly-purified samples obtained by HPLC purification of FAMEs. The technique described here increases effort of analysis by about 1 h per sample, but may be rewarding for challenging questions in fatty acid analysis.

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