Rapid separation of serum lipids for fatty acid analysis by a single aminopropyl column

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Summary A rapid and simple method for separation of serum lipid classes for fatty acid analysis with a single aminopropyl solid phase glass column is described. The recoveries of cholesteryl esters, triglycerides, free fatty acids, and phospholipids were all at least 98%. Coefficients of variation less than 10% were obtained for absolute and relative amounts of most individual fatty acids analyzed after separation of serum lipid classes. This method provides an efficient and convenient tool to follow fatty acid patterns in serum lipid fractions.— **Ågren, J. J., A. Julkunen, and I. Penttilä.** Rapid separation of serum lipids for fatty acid analysis by a single aminopropyl column. *J. Lipid Res.* 1992. **33:** 1871–1876.

Supplementary key words cholesteryl ester • phospholipid • triglyceride • gas-liquid chromatography

The fatty acid composition of serum lipids is a subject of continuous interest in several types of studies. The methods for the separation of serum lipid classes have been, however, quite laborious. The most widely used methods have been thin-layer chromatography (TLC) and column chromatography. The advantages of column chromatography when compared to TLC are the higher yields, convenience of the procedure, and smaller risk of autooxidation of polyunsaturated fatty acids. Kaluzny and co-workers (1) presented a novel method where aminopropyl solid phase columns were used to separate several lipid classes with high purity and small amounts of solvents. This method has been modified to separate only CE and TG (2) or neutral and acidic phospholipids (3). However, two columns were needed to separate the major serum lipids which increased analysis time and the consumption of materials. In addition, acidified solvents elute considerable amounts of contaminants from commercial prepacked plastic columns (4).

This paper describes a simplified method to separate serum CE, TG, FFA, and PL. These lipids were separated in a single aminopropyl solid-phase packed glass column with high recovery and moderate solvent volumes. The fatty acid content and composition of these lipids from serum samples were also analyzed to examine the variation of the entire procedure.

MATERIALS AND METHODS

Materials

All lipid standards and 14% boron trifluoride-methanol were obtained from Sigma Chemical Co. (St. Louis, MO) with the exception of cholesteryl heptadecanoate which was obtained from Nu-Chek-Prep Inc. (Elysian, MN). Glass columns (barrel of 5-ml syringe with Teflon Luer lock) were obtained from Hamilton Co. (Reno, NV) and miniature valves were from Supelco Inc. (Bellefonte, PA). Aminopropyl bonded silica sorbent (Bondesil™), stainless-steel frits (20 μm porosity, 9 mm diameter), Vac Elut vacuum elution apparatus, and silica TLC plates (Empore™, 3M) were purchased from Analytichem International (Harbor City, CA).

Preparation of samples

A standard lipid mixture was prepared to study the recovery of different lipid classes. A portion of this mixture used in one separation contained 500 μg cholesteryl stearate (CE-18:0), 300 μg tripentadecanoin (TG-15:0), 80 μg dipalmitolein (DG-16:0), 80 μg monomyristolein (MG-14:0), 100 μg oleic acid (FFA-18:1), and 400 μg phosphatidylcholine diheptadecanoyl (PC-17:0). These amounts were at least twofold higher than those found in 100 μl of normolipidemic serum. Two dilutions (1:2, 1:4) were made from this mixture to study the recoveries with "normal" and low concentrations of lipids. The recoveries of standard lipids containing polyunsaturated fatty acids were measured by adding 100 μg cholesteryl linoleate (CE-18:2), trilinolein (TG-18:2), linoleic acid (FFA-18:2), or 1-palmitoyl,2-linoleoyl-phosphatidylcholine (PC-18:2) into the standard lipid mixture. Fatty acid analyses were made from three portions of each standard lipid mixture. α-Linolenic acid methyl ester (25 μg) was added to these portions to measure the quantity of fatty acids. It was added also to each fraction collected by column chromatography to calculate the recoveries of lipid classes. Standard lipids were also analyzed separately to check their purity.

The distribution of standard lipids was measured in the presence of serum lipids. In these studies, cholesteryl heptadecanoate (CE-17:0) and TG-15:0 or pentadecanoic acid (FFA-15:0) and PC-17:0 were added (50 μg of each) to lipid extract of hyperlipidemic serum. Docosatrienoic acid

Abbreviations: CE, cholesteryl ester; TG, triglyceride; FFA, free fatty acid; PL, phospholipid; DG, diglyceride; MG, monoglyceride; PC, phosphatidylcholine; SM, sphingomyelin; TLC, thin-layer chromatography.

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methyl ester (25 μg) was added to fractions collected during the column procedure to calculate the distribution of recovered lipids.

Serum lipids were extracted by the method of Folch, Lees, and Sloane Stanley (5). The serum sample (100 μl) was vigorously shaken with 3 ml of chloroform–methanol 2:1 for 2 min. The protein precipitate was removed by centrifugation (2000 *g*, 10 min). Distilled water (750 μl) was added to the supernatant and the tube was shaken and allowed to settle. The separated chloroform layer was transferred to a new tube and evaporated to dryness under nitrogen. The lipid extract was dissolved in small amount of chloroform for storing or directly used for the column procedure. CE-17:0 (25 μg of 17:0), TG-15:0 (25 μg of 15:0), FFA-15:0 (2.5 μg), and PC-17:0 (25 μg of 17:0) were used as internal standards and they were added to serum samples in 100 μl of chloroform before the extraction.

Column procedure

Aminopropyl phase (about 500 mg) was packed into a glass column that was equipped with a stainless-steel frit on the bottom and on the sorbent. The bottom frit was tightened from the sides with Teflon tape. The column was connected to a vacuum apparatus via a miniature valve. The sorbent was changed after each separation due to ease and quickness of packing compared to washing of sorbents with uncertain reproducibility.

Before loading the sample, the column was treated with 0.6 ml of acetone–water 7:1 and washed with two 1-ml portions of hexane. The lipid sample was evaporated to complete dryness under a stream of nitrogen, dissolved in 200 μl of hexane–methyl tert-butylether–acetic acid 100:3:0.3 and applied to the column. The sample tube was washed with 100 μl of this same solvent and the solvent was added to the column. Cholesteryl esters were eluted with 5 ml of hexane and TG with 6 ml of hexane–chloroform–ethyl acetate 100:5:5. The third eluent was chloroform–2-propanol 2:1 (5 ml) and this fraction contained mono- and diglycerides. Thereafter, FFAs were eluted with 6 ml of chloroform–methanol–acetic acid 100:2:2. Finally, phospholipids were eluted with 6 ml of methanol–chloroform–water 10:5:4. The chloroform layer containing phospholipids was separated by adding 2 ml water and 1 ml chloroform into the last fraction. All fractions were collected in screw-cap tubes with a Teflon seal.

The contaminants eluted from the column were studied by running blanks using 300 μl of sample solvent as a sample. An internal standard (α-linolenic acid methyl ester) was added to collected fractions to calculate the amount of contaminants.

Thin-layer chromatography

The lipid samples were evaporated under nitrogen and dissolved in a small volume of chloroform. TLC plates were predeveloped with chloroform–methanol–acetic acid 100:10:2 and dried before running the samples. The samples were applied to the TLC plates as narrow bands under a stream of nitrogen. The TLC plates were then developed with hexane–diethylether–acetic acid 75:25:1 and dried under a nitrogen flow. A strip of plate containing standard lipids was cut off and stained with iodine vapor. The areas corresponding to the stained bands in this strip were cut off from the plate and placed in the tubes containing 3 ml chloroform–methanol 2:1. The lipids were extracted and the separated chloroform layers were used for fatty acid analyses.

Fatty acid analysis

Fractions obtained from the column or TLC procedure were evaporated to dryness under a stream of nitrogen. With the exception of the FFA fraction, 0.1 ml toluene and 0.5 ml BF_3-methanol were added to each tube. The tubes were tightly capped and placed into a block heater (100°C) for 60 min. After cooling, 0.8 ml distilled water and 0.8 ml hexane were added and the tubes were shaken.

TABLE 1. Recovery of standard lipids separated by aminopropyl solid phase columns

Lipid	Fraction[a] 1	2	3	4	5
			% recovery		
CE-18:0	99.2 ± 1.6	0.2 ± 0.1	0	0	0
TG-15:0	0.4 ± 0.2	100.8 ± 1.6	0.6 ± 0.1	0	0
DG-16:0	0	0	100.4 ± 1.1	0	0
MG-14:0	0	0	100.2 ± 2.1	0.9 ± 0.8	0
FFA-18:1	0	0	0	99.0 ± 2.1	0.7 ± 0.2
PC-17:0	0	0	0	0.3 ± 0.2	99.1 ± 3.6

Values are given as mean ± SD, n = 8.
[a]Fraction 1 solvent is hexane; fraction 2 solvent is hexane–chloroform–ethyl acetate 100:5:5; fraction 3 solvent is chloroform–2-propanol 4:1; fraction 4 solvent is chloroform–methanol–acetic acid 100:2:2; fraction 5 solvent is methanol–chloroform–water 10:5:4.

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TABLE 2. Recovery of standard lipids containing linoleic acid separated by aminopropyl solid phase columns

Lipid	Fraction[a] 1	2	3	4	5
			% recovery		
CE-18:2	99.8 ± 2.8	0.4 ± 0.4	0	0	0
TG-18:2	0.5 ± 0.2	98.9 ± 2.1	1.1 ± 0.1	0	0
FFA-18:2	0	0	0	99.2 ± 1.0	1.0 ± 0.5
PC-18:2	0	0	0	0.6 ± 0.2	98.5 ± 2.2

Values are given as mean ± SD, n = 5.
[a]See Table 1 for fraction solvents.

Hexane containing fatty acid methyl esters was directly taken for gas chromatographic analysis. The FFA fraction was treated similarly otherwise but all volumes were halved and heating time was only 10 min.

Fatty acid methyl esters were analyzed by a Carlo Erba Vega 6130 gas chromatograph (Carlo Erba Instruments, Milan, Italy) equipped with a 25 m × 0.32 mm i.d. (film thickness 0.25 μm) fused silica capillary column (NB-351, HNU-Nordion Ltd, Helsinki, Finland). The oven temperature was programmed from 60°C to 250°C at 10°C/min and held for 13 min. Injector and flame ionization detector temperatures were 250°C and 260°C, respectively. Helium was used as a carrier gas (4 ml/min) and the split ratio was 1:4. The response factors for individual fatty acids were determined by using fatty acid standard mixtures.

RESULTS AND DISCUSSION

The recoveries of standard lipids separated on aminopropyl columns are presented in **Table 1** and **Table 2.** At least 98% recovery of all lipids was obtained. Practically identical results were obtained also from standard mixtures with lipid content near normolipidemic serum (n = 5) and with low lipid content (n = 5). PC is the major phospholipid in serum and was therefore used in these examinations. Sphingomyelin (SM) and phosphatidylethanolamine (PE) were studied separately (250 μg of both, n = 3). The recoveries of SM were 0 and 99.8%, and of PE were 0.2 and 99.2% in fractions 4 and 5, respectively.

After successful separation of standard lipid mixtures, standard lipids were added to lipid extracts of hyperlipidemic serum (total cholesterol 7.9 mmol/l, TG 4.9 mmol/l) to test this procedure in practice. The distribution of standard lipids showed that the presence of other constituents in serum extract did not change the elution properties of these lipids (**Table 3**).

The separation of CE and TG was the critical step to obtain a single column procedure. It has been observed that any chloroform present in the sample solvent causes the elution of TG with CE (1). In contrast to this, we found that TG were retained in the column even when 10% chloroform in hexane was used in the elution. The sorbent was, however, about 2 years old and TG were completely eluted when fresh sorbent was used. Also the dehydration of older sorbent with acetone abolished its capacity to retain TG. We tried to change the properties of fresh sorbent by preconditioning the column with different acetone-water mixtures. We were able to get complete separation of CE and TG with several modifications but the reproducibility was not good enough. In the next step we substituted chloroform in sample solvent

TABLE 3. Percentage distribution of standard lipids (50 μg of each) added to lipid extract of hyperlipidemic serum[a] and separated by aminopropyl solid phase columns

Lipid	Fraction[b] 1	2	3	4	5
			% distribution		
CE-17:0	99.7 ± 0.1	0.3 ± 0.1	0	0	0
TG-15:0	0.5 ± 0.3	98.7 ± 0.4	0.8 ± 0.2	0	0
FFA-15:0	0	0	0	99.2 ± 0.1	0.8 ± 0.1
PC-17:0	0	0	0	0.4 ± 0.1	99.6 ± 0.1

Values are given as mean ± SD, n = 5.
[a]Total cholesterol 7.9 mmol/l; triglycerides 4.9 mmol/l.
[b]See Table 1 for fraction solvents.

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with methyl tert-butylether which has been successfully used with silica columns (6). Almost complete separation of CE and TG was obtained. In fact, no acetone-water treatment was needed but it was still used to improve the separation of FFA.

It has been shown that contaminants that co-chromatograph especially with saturated and monounsaturated fatty acids are eluted from plastic columns with acidified solvents (4). We observed also in the FFA fraction that much more fatty acid was found in the blank run than was present in 100 μl of serum when prepacked Bond Elut columns were used. Thus, we excluded all plastic material, using instead glass columns, stainless-steel frits, Teflon tubing in the elution apparatus, and glass pipettes. The extent of contamination in the FFA fraction was substantially decreased but not totally eliminated. The amounts of contaminants eluted in the FFA fraction were 1.3 ± 0.1 nmol (14:0), 5.4 ± 0.4 nmol (16:0), 0.5 ± 0.0 nmol (16:1n7), 4.4 ± 0.5 nmol (18:0), and 1.1 ± 0.1 nmol (18:1n9) (mean ± SD, n = 4).

A sample size of 100 μl of serum was sufficient for analysis of fatty acids in CE, TG, and PL fractions. The amounts of FFA were so small that the methylation step was slightly modified to obtain a more concentrated sample. This was first made by evaporation of the solvent under a stream of nitrogen. We found, however, that even with a gentle nitrogen stream, 14-18 -carbon fatty acid methyl esters started to evaporate. For example, when 1 ml of fatty acid standard mixture (25-50 μg individual fatty acids) in hexane was evaporated to dryness (15 min at 23°C) and instantly dissolved, about 18% of 14:0, 11% of 16:0, and 5% of 18:0 were evaporated. Therefore, the volumes in the methylation step were planned so that the sample for gas chromatography was directly obtained.

Ten 100-μl aliquots of the same serum sample were analyzed to determine the precision of the method. The fatty acid content and composition of the major serum lipid classes are presented in **Table 4.** The coefficients of variation were from 0.9 to 15.9% for the relative amounts and from 3.0 to 14.8% for the absolute amounts of fatty acids and were mostly under 10% in both cases. The variations of FFA were in general slightly greater than in the other fractions. This is not surprising when considering their small amounts and the use of blank values. It should be noted, however, that the contaminants of this fraction were quite constant and polyunsaturated fatty acids were not affected. This is an advantage when compared to direct methylation methods of FFA (7, 8). In the latter methods, already very small (1-2%) transmethylation of other lipids would give considerable nonspecific rise of FFA (30-60 μg/ml in our normolipidemic test sample) which would also be dependent on the total lipid content. On the other hand, possible liberation of fatty acids from other fractions and difficulties with gas-liquid chromatography limit the usefulness of direct measurement of FFA without derivatization (9).

The precision of the column procedure was compared with TLC (**Table 5**). Several serum lipid extracts were

TABLE 4. Fatty acid content and composition (mol % of total) in cholesteryl esters (CE), triglycerides (TG), free fatty acids (FFA), and phospholipids (PL) and their coefficients of variation (CV, %) obtained from analyses of ten 100-μl aliquots of normolipidemic serum

	CE		TG		FFA		PL	
Fatty Acid	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV
	mol %		*mol %*		*mol %*		*mol %*	
14:0	1.2 ± 0.1	4.5	5.5 ± 0.2	3.6	5.7 ± 0.5	9.5	0.9 ± 0.1	8.1
16:0	10.9 ± 0.2	1.5	28.5 ± 0.5	1.9	31.0 ± 0.8	2.7	29.6 ± 0.5	1.7
18:0	1.0 ± 0.1	10.9	4.3 ± 0.3	6.2	17.5 ± 0.9	5.0	12.3 ± 0.3	2.5
20:0							0.4 ± 0.0	5.5
22:0							1.0 ± 0.1	5.8
24:0							0.8 ± 0.1	5.3
16:1n-7 + n-9	3.0 ± 0.1	3.7	5.9 ± 0.3	4.9	4.7 ± 0.7	15.9	0.9 ± 0.1	8.8
18:1n-9	16.7 ± 0.3	1.8	34.0 ± 0.5	1.4	23.4 ± 1.2	5.1	9.7 ± 0.1	1.2
18:1n-7	1.4 ± 0.1	5.3	2.7 ± 0.2	8.9	2.2 ± 0.2	9.5	1.7 ± 0.1	4.0
24:1n-9							2.1 ± 0.1	5.7
18:2n-6	54.8 ± 0.5	0.9	12.9 ± 0.3	2.6	10.5 ± 0.7	6.2	22.4 ± 0.3	1.3
18:3n-6	0.9 ± 0.0	1.3	0.7 ± 0.0	3.2			0.2 ± 0.0	10.1
18:3n-3	0.9 ± 0.0	1.6	1.0 ± 0.0	3.9	1.4 ± 0.1	8.0	0.3 ± 0.0	5.1
20:3n-6	0.5 ± 0.0	5.3					2.2 ± 0.1	2.6
20:4n-6	6.2 ± 0.2	3.9	1.7 ± 0.1	4.1	1.3 ± 0.1	9.0	7.7 ± 0.2	2.7
20:5n-3	1.7 ± 0.1	3.9	0.6 ± 0.0	6.7			1.8 ± 0.0	2.3
22:4n-6							0.5 ± 0.0	8.2
22:5n-3			0.6 ± 0.0	5.8	0.9 ± 0.1	11.1	0.9 ± 0.1	6.3
22:6n-3	0.8 ± 0.1	5.7	1.6 ± 0.1	4.7	1.4 ± 0.1	9.5	4.6 ± 0.2	3.2
Total amount (mmol/l)	4.32 ± 0.12	2.9	1.84 ± 0.07	3.7	0.38 ± 0.01	3.5	4.55 ± 0.16	3.5

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TABLE 5. Fatty acid content and relative amounts of some major fatty acids (mol % of total) in cholesteryl esters (CE), triglycerides (TG), free fatty acids (FFA), and phospholipids (PL) and their coefficients of variation (CV, %) determined after separation of same serum lipid extract by column chromatography (CC) or thin-layer chromatography (TLC) (n = 6)

Fatty Acid	CC		TLC	
	Mean ± SD	CV	Mean ± SD	CV
CE				
16:0	11.1 ± 0.2	1.5	11.5 ± 0.3	2.7
18:1n-9	21.4 ± 0.1	0.4	22.2 ± 0.8	3.4
18:2n-6	48.4 ± 0.1	0.2	48.5 ± 0.3	0.6
20:4n-6	6.0 ± 0.2	2.5	4.9 ± 0.6	11.5
22:6n-3	0.6 ± 0.0	3.9	0.4 ± 0.1	14.1
Total amount[a] (mmol/l)	6.33 ± 0.09	1.5	5.72 ± 0.32	5.5
TG				
16:0	28.0 ± 0.3	1.2	29.2 ± 0.3	0.9
18:1n-9	40.5 ± 0.3	0.7	41.1 ± 0.8	2.0
18:2n-6	10.0 ± 0.3	3.0	9.0 ± 0.3	3.1
20:4n-6	1.0 ± 0.1	7.2	0.8 ± 0.0	2.9
22:6n-3	0.5 ± 0.0	3.8	0.4 ± 0.0	8.3
Total amount/3[b] (mmol/l)	1.47 ± 0.02	1.6	1.37 ± 0.03	1.9
FFA				
16:0	30.0 ± 0.4	1.5	29.8 ± 0.6	1.9
18:1n-9	33.4 ± 0.8	2.5	34.2 ± 0.4	1.3
18:2n-6	7.5 ± 0.2	2.6	7.7 ± 0.4	4.9
20:4n-6	0.6 ± 0.0	7.8	0.6 ± 0.0	3.6
22:6n-3	0.4 ± 0.0	8.3	0.4 ± 0.1	14.5
Total amount (mmol/l)	0.86 ± 0.01	1.7	0.83 ± 0.01	1.8
PL				
16:0	29.3 ± 0.6	2.2	29.1 ± 0.4	1.4
18:1n-9	11.3 ± 0.2	1.9	10.8 ± 0.2	2.1
18:2n-6	21.2 ± 0.2	0.8	20.8 ± 0.1	0.4
20:4n-6	7.9 ± 0.2	2.8	8.1 ± 0.2	2.1
22:6n-3	3.7 ± 0.2	4.6	3.9 ± 0.1	2.7
Total amount (mmol/l)	6.96 ± 0.05	0.7	7.05 ± 0.08	1.1

[a]6.39 mmol/l when determined by enzymatic method (total cholesterol minus free cholesterol) (Boehringer Mannheim, Mannheim, Germany).
[b]1.53 mmol/l when determined by enzymatic method (Boehringer Mannheim, Mannheim, Germany).

made from the same sample and these extracts were pooled to avoid variation caused by the pipetting of serum sample and standard mixture. The clearest difference between these methods was seen for CE. The coefficients of variation were greater and the total amount of fatty acids was smaller when determined with TLC. In addition, the proportions of saturated and monounsaturated fatty acids were slightly greater in the TLC-processed samples. This was observed also for TG. No significant differences were found in the fatty acid content and composition of FFA and PL between these methods. With the column procedure, the total amount of CE was 99.1% and of TG 96.1% of that obtained with enzymatic methods. This percentage was 89.5 for both CE and TG when the TLC method was used. These results show that similar or more precise results can be obtained with the more convenient procedure of solid phase extraction than TLC. It should also be noted that the amounts of fatty acids obtained for the gas chromatographic analyses were clearly greater after the column than the TLC separation.

At the moment, contamination caused by the plastic materials limits the usefulness of commercial prepacked columns. However, they can be used when the fatty acid compositions of CE or TG are analyzed. Also, the polyunsaturated fatty acids of FFA and PL can be measured without disturbing contaminants.

The present method offers a rapid and economical separation of serum lipid classes with high recovery and purity. Direct separation of CE and TG makes it now possible to isolate all major serum lipid classes using a single aminopropyl solid phase column. This simplifies and shortens the method and makes it also more economical as compared to earlier applications.

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