Journal of Chromatography, 564 (1991) 272–277
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5671

Short Communication

Simple thin-layer chromatographic purification procedure for the determination of cholesterol ester fatty acid compositions

CORNELIUS M. SMUTS and HENDRIK Y. TICHELAAR*

Research Institute for Nutritional Diseases, South African Medical Research Council, P.O. Box 70, Tygerberg 7505 (South Africa)

(First received July 31st, 1990; revised manuscript received October 8th, 1990)

ABSTRACT

A mild procedure for the purification of methyl esters of the fatty acid components of cholesterol esters, from interfering free cholesterol and other contaminating residues, is described. Methyl esters and free cholesterol are formed during the methylation of cholesterol esters. When co-extracted, cholesterol and other contaminating residues interfere with the methyl esters because minute proportions of these residues tend to elute at the same retention times as palmitoleic and stearic acids, to yield unreliable but significantly higher values for palmitoleic ($p < 0.001$) and stearic acids ($p < 0.0001$), and correspondingly lower values for oleic acid ($p < 0.0001$). Purification of methyl esters by thin-layer chromatography eliminates this problem and yields reliable analysis of cholesterol ester fatty acids, without measurable oxidation of unsaturated fatty acids.

INTRODUCTION

Cholesterol esters occur naturally in plasma lipoproteins and accumulate in blood vessel walls as fatty streaks in atherosclerosis [1,2]. Cholesterol ester fatty acid composition analysis is conventionally done by gas chromatography (GC) after the lipids are extracted by either a modification of the Folch procedure [3] or by Dole's procedure [4–6], followed by thin-layer chromatography (TLC) [3,7], or a procedure with silica Sep-Pak cartridges [8]. During hydrolysis and methylation, methyl esters and free cholesterol are formed from cholesterol esters. This cholesterol and other contaminating residues are co-extracted with the methyl esters and interfere with the GC analysis, particularly of palmitoleic and stearic acids, by amplifying their percentage composition values. Long-chain fatty acids, especially the C_{22} fatty acids, should also be involved because free cholesterol elutes at approximately the same retention times as these fatty acids. The aim of this study was to improve the GC analysis of cholesterol esters, using a rapid and reliable modification of an existing TLC procedure [7].

0378-4347/91/$03.50

EXPERIMENTAL

Samples

Venous blood was collected from a group of seven healthy human subjects, into tubes containing potassium EDTA (1 mg/ml). Plasma was prepared by centrifugation (1520 *g*) at 4°C for 15 min. All plasma was pooled and stored at 4°C until used in the study.

Methods

Aliquots ($n=30$) of the pooled plasma were extracted by Dole's procedure [4–6], and the hexane phases were submitted to TLC after drying under nitrogen. Duplicate spots were made of each sample [7] on 10 × 10 cm pre-coated TLC plates containing silica gel 60 without a fluorescent indicator (Art. 5721, Merck, Darmstadt, Germany). The TLC plates were developed in small filter paper-lined glass tanks containing petroleum ether–diethyl ether (peroxide free)–acetic acid (90:30:1, v/v).

Plates were dried under a stream of nitrogen for 20 min and sprayed with chloroform–methanol (1:1, v/v) containing $232 \cdot 10^{-6}$ *M* BBOT [2,5-bis-2-(5-*tert.*-butylbenzoxazolyl)thiophene]. Cholesterol esters were observed under UV light (365 nm), after being identified from R_F values (cholesterol esters, triacylglycerol, free fatty acids and free cholesterol at 0.81, 0.59, 0.24 and 0.12, respectively). Areas containing the cholesterol esters were scraped into glass-stoppered tubes and transmethylated for 3 h at 70°C with 3 ml of methanol–11.6 *M* sulphuric acid (95:5, v/v). After cooling, 1 ml of distilled water was added and the reaction products (mainly fatty acid methyl esters and free cholesterol) were extracted with 2 ml of hexane. The hexane phase was then transferred to a separate glass-stoppered tube and evaporated to dryness under a stream of nitrogen. One of the duplicate samples was then dissolved in carbon disulphide and subjected to GC analysis. At least 30 min at 240°C had to be allowed between analyses to prevent late eluting peaks from interfering with the next chromatographic run. The second duplicate sample was dissolved in chloroform–methanol–0.15 *M* NaCl (86:14:1, v/v) and chromatographed by TLC in petroleum ether–diethyl ether–acetic acid as described above. Areas containing the methyl esters were visualized as before (R_F values for methyl esters and free cholesterol are 0.60 and 0.12, respectively), scraped into glass-stoppered tubes and suspended in 3 ml of methanol. This fraction contained no free cholesterol because the methyl esters were well separated from the cholesterol and other contaminating residues on the TLC plate. To this was added 1 ml of distilled water, and the methyl esters were subsequently extracted with 2 ml of hexane. The hexane phase was evaporated to dryness and submitted to GC analysis [7].

Gas chromatography

Fatty acid methyl ester analysis was performed on a Varian Model 3700 gas

chromatograph equipped with a flame ionization detector and a Varian Model CDS-402 data system. Separations were done on a 1.8 m × 1.9 mm I.D. glass column, packed with 10% SP-2330 on 100–120 mesh Chromosorb W AW (Supelco, Cat. No. 1-1851). The column starting temperature was 150°C and was programmed to 220°C at 3°C/min. The injector temperature was 240°C and the detector temperature was 250°C. Nitrogen was used as the carrier gas at a flow-rate of 25 ml/min.

Percentage fatty acid composition

Duplicate samples were analysed following methylation and re-spotting. All the lipid components were subjected to GC analysis. The results are expressed as percentages of the total area of all fatty acid peaks, from palmitic acid to arachidonic acid.

Reproducibility

Sample reproducibility of the percentage composition of fatty acids in plasma cholesterol esters was analysed on eleven samples from the plasma pool, which were purified from free cholesterol and other contaminating residues by re-submitting to TLC (Table I).

Statistical analysis

The Wilcoxon paired rank test was used to compare data of the duplicate samples before and after re-spotting.

RESULTS

The fatty acid composition of cholesterol esters was determined in thirty duplicate aliquots from the plasma pool after exclusion and inclusion of a second TLC step (Table II). When free cholesterol and other contaminating residues were not removed by means of a second TLC step, palmitoleic acid was significantly higher

TABLE I

SAMPLE REPRODUCIBILITY OF THE PERCENTAGE COMPOSITION OF FATTY ACIDS IN PLASMA CHOLESTEROL ESTERS

Fatty acid		Mean ± S.D. ($n = 11$)	C.V. (%)
$C_{16:0}$	Palmitic acid	10.15 ± 0.59	5.8
$C_{16:1}$	Palmitoleic acid	3.05 ± 0.37	12.1
$C_{18:0}$	Stearic acid	1.14 ± 0.15	13.2
$C_{18:1}$	Oleic acid	19.34 ± 0.56	2.9
$C_{18:2}$	Linoleic acid	60.04 ± 1.31	2.2
$C_{20:4}$	Arachidonic acid	6.43 ± 0.43	6.7

TABLE II

COMPARISON OF PERCENTAGE FATTY ACIDS IN CHOLESTEROL ESTERS AFTER EXCLUSION AND INCLUSION OF A SECOND TLC STEP

Fatty acid		Excluding second TLC (mean ± S.D., (n = 30)	C.V. (%)	Including second TLC (mean ± S.D.)[a]	C.V. (%)	Statistical significance
$C_{16:0}$	Palmitic acid	10.72 ± 0.39	3.6	10.68 ± 0.80	8.1	
$C_{16:1}$	Palmitoleic acid	3.66 ± 0.35	9.6	3.24 ± 0.44	13.6	–[a]
$C_{18:0}$	Stearic acid	1.32 ± 0.36	27.3	0.85 ± 0.14	16.5	–[b]
$C_{18:1}$	Oleic acid	18.80 ± 0.50	2.7	19.45 ± 0.82	4.2	–[b]
$C_{18:2}$	Linoleic acid	59.08 ± 1.08	1.8	59.53 ± 1.50	2.5	
$C_{20:4}$	Arachidonic acid	6.31 ± 0.47	7.4	6.29 ± 0.78	12.4	

[a] $p < 0.001$
[b] $p < 0.0001$

($p < 0.001$), while stearic acid was even higher ($p < 0.0001$). Oleic acid, however, was significantly lower ($p < 0.0001$). Fig. 1 shows the cholesterol and contaminating residue peaks, excluding (Fig. 1a) and including (Fig. 1b) TLC purification in the same sample.

The mean percentage composition of fatty acids and the coefficient of variation (C.V.) of each fatty acid determination of the plasma cholesterol esters of eleven plasma samples from the plasma pool are shown in Table I.

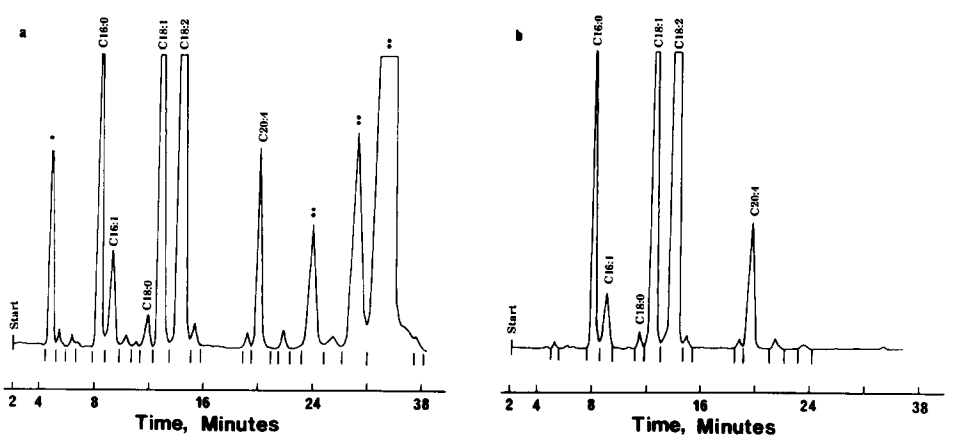

Fig. 1. Chromatogram of pooled plasma aliquots (a) excluding and (b) including TLC purification, showing the effects of contaminating interference peaks. Peaks: * = BHT [2,5-bis-2-(5-*tert.*-butylbenzoxazolyl)-thiophene]; ** = FC (free cholesterol), cholestadiene, cholesterol ether and other by-products.

DISCUSSION

Cholesterol esters are formed when cholesterol and a fatty acid are linked via an ester bond at the steroid 3-position. If contaminating residues, including cholesterol, cholestadiene, cholesterol ether and other byproducts, are not removed after methylation of cholesterol esters, or when insufficient time is allowed between GC analyses to purify the columns, unreliable fatty acid analysis results may be produced. Subsequent to column purification, minute proportions of these residues still tend to elute at the same retention times as palmitoleic and stearic acids, and this increases the apparent percentage composition of these fatty acids. It should be noted that if the columns are inadequately purified between analyses, contamination of these fatty acids is less ambiguous.

Methyl esters can be prepared by removing cholesterol with the more vigorous saponification–reesterification procedure, in which the preliminary saponification hydrolyses the cholesterol from cholesterol esters, but results in a prolonged hydrolysis time that may augment oxidation of the fatty acids [9–11]. Alternatively, a transesterification procedure followed by TLC purification [2,7,12] can be used to overcome this problem. Otherwise, sufficient time can be allowed between GC analyses: a minimum of 30 min at 240°C is necessary, however, this reduces the number of samples that can be analysed per day. When cholesterol has been removed prior to GC analysis, 10 min equilibration time is sufficient between injections.

Although interference by cholesterol and other contaminating residues results in false high values for palmitoleic and stearic acids, and correspondingly low values for oleic acid, neither linoleic nor arachidonic acid is affected. This implies that re-submitting of methyl esters to a second TLC step after methylation does not result in the oxidation of these two fatty acids to any measurable extent.

Cholesterol interference peaks appear at nearly the same retention times as for docosapentaenoic acid ($C_{22:5}$) and docosahexaenoic acid ($C_{22:6}$) (unpublished results). Since the analysis of these fatty acids is important in fish oil studies, cholesterol removal is imperative. Cholesterol esters are major constituents of the core of lipoprotein particles, and consequently interference by free cholesterol and other contaminating residues in fatty acid analysis may also lead to incorrect interpretations of lipoprotein compositional studies.

The sample reproducibility of fatty acid analysis (Table I) yielded very similar results to those obtained by other authors [10,13], who used the saponification–reesterification procedure. It should be mentioned that our TLC procedure described previously [7] has proved to give better reproducibility. Against the background of the consistency and reproducibility of the fatty acid analysis demonstrated in the present study (Table II), even minor interferences should be excluded as these could lead to significant compositional changes in fatty acid analysis.

Although a second TLC purification step does not necessarily yield improved

analyses, twice as many samples can be analysed per day and C_{22} fatty acids can be analysed with confidence.

Considering the speed and accuracy of our TLC procedure, we recommend that cholesterol and other contaminating residues should be removed after methylation to yield rapid and reliable results for cholesterol ester fatty acid compositions.

ACKNOWLEDGEMENTS

We thank Mrs. Renette J. Blignaut for statistical support, Mr. M. J. Van Wyk for photographic support, the blood donors, Dr. M. A. Dhansay for the blood collections and Dr. P. G. Thiel for his editorial comments.

REFERENCES

1 A. J. S. Benadé, J. E. Fincham, C. M. Smuts, M. T. Lai Tung, D. Chalton, M. Kruger, M. J. Weight, A. K. Daubitzer and H. Y. Tichelaar, *Atherosclerosis*, 74 (1988) 157.
2 B. Lundberg, *Atherosclerosis*, 56 (1985) 93.
3 D. E. Bowyer and J. P. King, *J. Chromatogr.*, 143 (1977) 73.
4 V. P. Dole and H. Meinertz, *J. Biol. Chem.*, 235 (1960) 259.
5 V. P. Dole, *J. Clin. Invest.*, 35 (1956) 150.
6 V. P. Dole, A. T. James, J. P. W. Webb, M. A. Rizack and M. F. Sturman, *J. Clin. Invest.*, 38 (1959) 1544.
7 H. Y. Tichelaar, A. J. S. Benadé, A. K. Daubitzer and T. J. Van Wyk Kotze, *Clin. Chim. Acta*, 183 (1989) 207.
8 S. T. Wang and F. Peter, *J. Chromatogr.*, 276 (1983) 249.
9 T. Moilanen and T. Nikkari, *Clin. Chim. Acta*, 114 (1981) 111.
10 T. Moilanen, *Lipids*, 22 (1987) 250.
11 J. MacGee and K. G. Allen, *J. Chromatogr.*, 100 (1974) 35.
12 R. C. Tuckey and P. M. Stevenson, *Biochemistry*, 94 (1979) 402.
13 M. Höckel, W. Dunges, A. Holzer, P. Brockerhoff and G. H. Rathgen, *J. Chromatogr.*, 221 (1980) 205.