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GAS—LIQUID CHROMATOGRAPHIC DETERMINATION OF FATTY ACID COMPOSITION OF CHOLESTERYL ESTERS IN HUMAN SERUM USING SILICA SEP-PAK CARTRIDGES

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SUMMARY

A simple and fast analytical procedure for separation and purification of cholesteryl esters of human serum is described. A single lipid extract, together with spiked cholesteryl pentadecanoate, as an internal standard, was passed through a Silica Sep-Pak cartridge. 1.5% diethyl ether in light petroleum was used to elute cholesteryl esters from the column. The separation was verified with thin-layer chromatography on silica gel using light petroleum—diethyl ether—glacial acetic acid (80:20:1) as a solvent. A very clean thin-layer chromatogram of cholesteryl esters without any additional spots of other lipids was obtained. The cholesteryl esters were quantitated by analyzing their fatty acid composition as methyl esters by gas—liquid chromatography. The coefficients of variation were 0.8—4.9% for the major fatty acids ($C_{16:0}$, $C_{16:1}$, $C_{18:1}$, $C_{18:2}$, $C_{20:4}$) and 6.7—30.8% for the minor fatty acids ($C_{18:0}$ and $C_{20:0}$). The recoveries for cholesteryl palmitate, cholesteryl oleate and cholesteryl linoleate were 90.7, 92.3 and 91.0%, respectively.

INTRODUCTION

Cholesteryl esters are normal constituents of lipoproteins in the blood and are the principal lipids to accumulate in the arterial wall during the progress of atherosclerosis [1]. They represent 60—75% of total cholesterol in the plasma in a normal healthy person. While the measurement of serum total cholesterol is one of the most frequently performed assays in the clinical laboratory, the analysis of cholesteryl esters, for their fatty acid composition, is not as widespread. The determination of the fatty acid composition of cholesteryl esters in plasma or serum is conventionally done by gas—liquid chromatography (GLC) after the lipids are extracted and the individual fractions separated by thin-layer chromatography (TLC) [2] or column chromatography [3, 4].

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There are drawbacks of both methods: The TLC procedure, which involves separation and extraction of the scraped-off fractions, is lengthy and cumbersome, and the column chromatography requires a large amount of sample. Recently Waters Assoc. have introduced a variety of small Sep-Pak cartridges for sample clean-up. These Sep-Pak cartridges are particularly useful in the clinical laboratory since they require only a small sample size. We are reporting here the investigation of the use of the silica Sep-Pak cartridges in combination with GLC for the determination of fatty acid composition of cholesteryl esters.

EXPERIMENTAL

Materials

The silica Sep-Pak cartridges were purchased from Waters Assoc. (Mississauga, Canada). Fatty acid methyl esters of PolyScience (Niles, IL, U.S.A.) and boron trifluoride—methanol of Pierce (Rockford, IL, U.S.A.) were obtained through Chromatographic Specialties (Brockville, Canada). Pentadecanoic acid methyl ester, cholesteryl pentadecanoate and cholesteryl esters were obtained from Sigma (St. Louis, MO, U.S.A.). The GLC glass column, packed with 10% SP-2330 on Chromosorb W AW 100—200 mesh, and the AOCS oil reference mixture of RM-3, were purchased from Supelco (Bellefonte, PA, U.S.A.). The E. Merck precoated TLC plates, silica gel 60, were supplied by BDH (Toronto, Canada), and Rhodamine 6G was obtained from Fisher Scientific (Toronto, Canada). All solvents were of analytical grade.

Lipid extraction

To 0.1 ml of serum and 10 μg of cholesteryl pentadecanoate as an internal standard, dissolved in 20 μl of chloroform, was added a mixture of 2 ml of isopropranol and 1 ml of water,. The sample was mixed on a Vortex mixer for 30 sec and then 2 ml of *n*-octane were added; this mixture was vortexed for another 30 sec. Brief centrifugation separated the upper octane layer which was then removed and evaporated to dryness at 37°C in a stream of nitrogen. The method of Folch et al. [5] was also used to extract 0.1 ml of serum with 5 ml of a chloroform—methanol (2:1) mixture. After phase separation the upper water–methanol layer was aspirated and the lower layer was washed with 0.2 volume of distilled water. The chloroform layer was removed and evaporated under a stream of nitrogen.

Silica Sep-Pak separation

Hexane (0.2 ml) was added to the dry extract and the solution was applied to the Sep-Pak column using a Pasteur pipette. An additional 0.2 ml of light petroleum (b.p. 20—40°C) was used to rinse the residue left in the test tube and this solution was again passed through the column. The Sep-Pak column was filled with 5 ml of 1.5% diethyl ether in light petroleum. The eluted sample was collected and then evaporated to dryness at 37°C under a nitrogen stream.

Esterification

A modification of a method described by Metcalfe and Schmitz [6] was used for saponification and methylation. A 0.5-ml volume of a 0.5 *M* sodium

hydroxide—methanol solution was added to the dried sample, purged with nitrogen and then heated in a heating block for 15 min at 100°C. Boron trifluoride in methanol (0.5 ml) was added and the mixture was heated for an additional 10 min at 100°C. The reaction mixture was cooled, extracted two times with 1.5 ml of light petroleum, and evaporated to dryness at 37°C in small vial under a stream of nitrogen. The residue was dissolved in 10 μl of *n*-heptane, of which 1 μl was introduced into the injection port of the gas—liquid chromatograph.

TLC separation

To prove the effectiveness of the Sep-Pak separation, the separated fraction from the Sep-Pak column was chromatographed on a TLC plate with a mixture of light petroleum—diethyl ether—glacial acetic acid (80:20:1) as solvent. The TLC plate was sprayed with aqueous sulfuric acid and charred for 10 min at 200°C. For comparison, the conventional TLC separation of the lipid extract was chromatographed and the bands made visible under UV light by spraying the plate with the Rhodamine 6G solution.

The corresponding band of cholesteryl esters on the TLC plate was scraped off and extracted three times with chloroform. The extracted cholesteryl esters were hydrolyzed and the fatty acids methylated and analyzed as described.

GLC determination

A Hewlett-Packard 5830A gas chromatograph, equipped with a hydrogen flame ionization detector, a Hewlett-Packard 18850A GC microprocessor terminal, and a single glass column of 183 cm × 6.4 mm O.D. (2 mm I.D.) packed with 10% SP-2330 on Chromosorb W AW, 100—120 mesh, was used. The column temperature was 200°C, the flow-rate of the nitrogen carrier gas was 20 ml/min.

RESULTS

The identification of cholesteryl esters, after being eluted from the Sep-Pak cartridge, was performed with the TLC system. It showed a very clean thin-layer chromatogram of cholesteryl esters without any additional spot, both with the lipid extraction method of Folch et al. [5] and with our *n*-octane—isopropanol—water procedure (Fig. 1). Both extraction methods were suitable for the Sep-Pak procedure. For the quantitative determination of the fatty acid composition of cholesteryl esters, they were hydrolyzed, methylated and chromatographed by GLC after being eluted from the Sep-Pak cartridge or scraped from a TLC plate. A typical gas—liquid chromatogram of fatty acids of human serum cholesteryl esters is shown in Fig. 2. The linearity of the detector response to different fatty acid methyl esters in the investigated concentration range was established, using pure standards.

The reproducibility of the method was estimated by carrying out six replicates on one serum sample as indicated in Table I. The average analytical coefficient of variation (C.V.), due to the instrument (GLC response) itself, was 1.9%. The coefficient of variation for the within-day assay and the between-day assay was 0.8—4.9% for the major fatty acids ($C_{16:0}$, $C_{16:1}$,

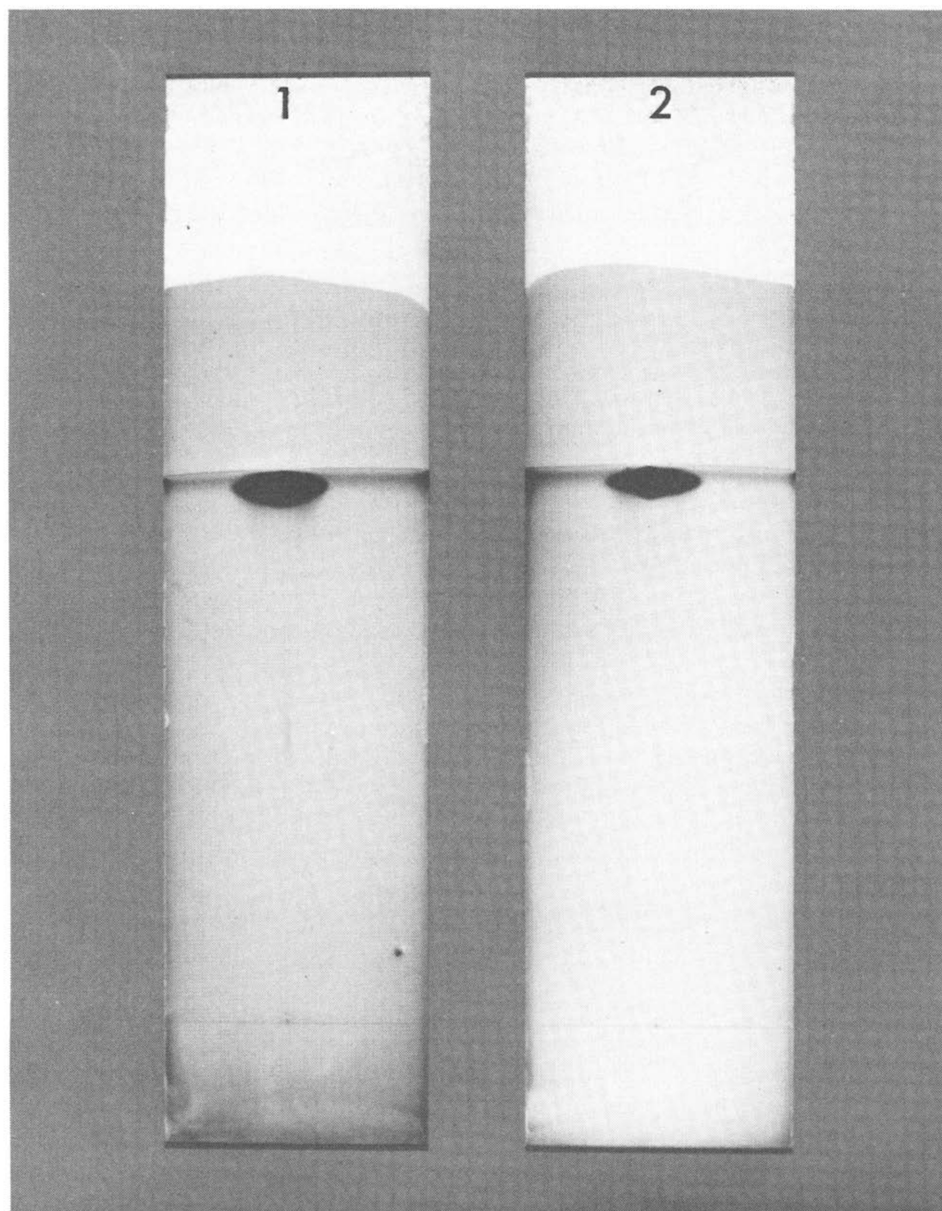

Fig. 1. A clean thin-layer chromatogram of cholesteryl esters after elution from Sep-Pak cartridges: (1) with *n*-octane—isopropanol—water lipid extraction; (2) with chloroform—methanol lipid extraction.

$C_{18:1}$, $C_{18:2}$ and $C_{20:4}$) and 6.7—30.8% for the minor fatty acids (less than 2% of the total, $C_{18:0}$ and $C_{20:0}$). There were no statistically significant differences between the within-day and between-day assay variations. The recovery was determined by assaying a serum sample after the addition of a known amount of cholesteryl ester standards of cholesteryl palmitate, cholesteryl oleate and cholesteryl linoleate. The percent recoveries for the method are shown in

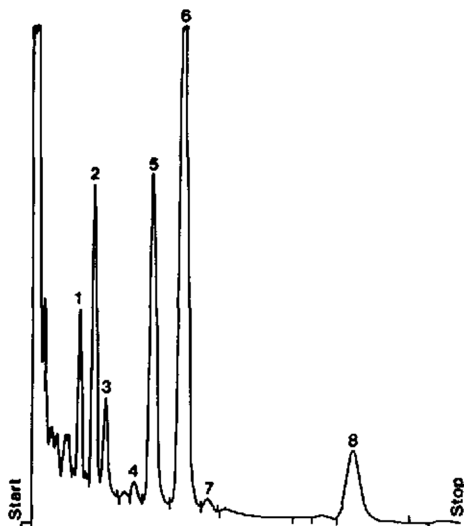

Fig. 2. Gas—liquid chromatogram of fatty acids of human serum cholesteryl esters. Peaks: 1 = $C_{15:0}$, internal standard; 2 = $C_{16:0}$; 3 = $C_{16:1}$; 4 = $C_{18:0}$; 5 = $C_{18:1}$; 6 = $C_{18:2}$; 7 = $C_{20:0}$; 8 = $C_{20:4}$.

TABLE I

PRECISION OF GLC MEASUREMENT OF FATTY ACID COMPOSITION OF CHOLESTERYL ESTERS (n = 6)

Fatty acid	Total fatty acid (%)					
	GLC response (mean ± S.D.)	C.V. (%)	Within-day precision (mean ± S.D.)	C.V. (%)	Day-to-day precision (mean ± S.D.)	C.V. (%)
$C_{16:0}$	11.9 ± 0.2	2.0	11.9 ± 0.2	2.0	12.1 ± 0.3	2.5
$C_{16:1}$	5.0 ± 0.1	2.0	4.9 ± 0.1	2.6	5.2 ± 0.2	3.8
$C_{18:0}$	1.1 ± 0.05	0.5	1.2 ± 0.2	16.6	1.3 ± 0.4	30.8
$C_{18:1}$	22.8 ± 0.1	0.4	22.7 ± 0.6	2.6	22.7 ± 0.9	4.0
$C_{18:2}$	48.5 ± 0.2	0.4	48.4 ± 0.4	0.8	47.9 ± 0.9	1.9
$C_{20:0}$	0.7 ± 0.05	7.1	0.6 ± 0.04	6.7	0.7 ± 0.1	14.3
$C_{20:4}$	10.1 ± 0.1	1.0	10.3 ± 0.3	2.9	10.3 ± 0.5	4.9

TABLE II

RECOVERY OF CHOLESTERYL ESTERS BY THE SEP-PAK CARTRIDGE WITH DIFFERENT LIPID EXTRACTION

	Recovery (%)		
	Chloroform—methanol	Hexane—methanol	n-Octane—isopropanol—water
Cholesteryl palmitate	90.7	88.7	92.8
Cholesteryl oleate	92.3	88.6	94.2
Cholesteryl linoleate	91.0	87.6	92.8

Table II. Both extraction methods of n-octane—isopropanol—water and chloroform—methanol showed more than 90% recoveries. The slightly increased recovery with the octane extraction method may be due to the fact that the cholesteryl esters are highly lipophilic.

To check further that our Sep-Pak method measured the true fatty acid composition of cholesteryl esters, we compared our procedure with the conventional TLC method. The result of this study is shown in Table III. There were no significant differences between our Sep-Pak method and the conventional TLC method. The results also showed that the lipid extraction of Folch et al. [5] (chloroform—methanol) can also be used in conjuction with the Sep-Pak column as indicated previously on the qualitative identification. The validity of the Sep-Pak method was further checked on six serum specimens with cholesterol and triglyceride concentrations ranging from 172—296 and 135—282 mg/dl, respectively. As shown in Table IV, the results on the comparison of the Sep-Pak and TLC methods were in good agreement. The value of $C_{18:0}$ minor fatty acid of specimen No. 3 on the Sep-Pak method was slightly higher than the value of the TLC method. This was caused by the unresolved small peak on the gas chromatogram.

TABLE III

FATTY ACID COMPOSITION OF CHOLESTERYL ESTER DETERMINED BY DIFFERENT EXTRACTION METHODS (n = 6)

Values are given as percent total fatty acids (mean ± S.D.).

Fatty acid	Method		
	n-Octane—isopropanol—water (Sep-Pak)	Chloroform—methanol (Sep-Pak)	Chloroform—methanol (TLC)
$C_{16:0}$	11.9 ± 0.4	11.9 ± 0.3	11.3 ± 0.4
$C_{16:1}$	4.9 ± 0.4	5.1 ± 0.1	4.9 ± 0.1
$C_{18:0}$	1.2 ± 0.2	1.1 ± 0.3	0.8 ± 0.1
$C_{18:1}$	22.7 ± 0.6	22.9 ± 0.3	22.6 ± 0.4
$C_{18:2}$	48.4 ± 0.4	48.4 ± 0.4	49.1 ± 0.4
$C_{20:0}$	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
$C_{20:4}$	10.3 ± 0.3	10.0 ± 0.5	10.6 ± 0.3

DISCUSSION

A simple procedure has been developed by Peter and Reynolds [7] for the separation of cholesteryl esters from the other lipid components of serum. Cholesteryl esters were extracted into n-octane from a mixture of serum and alkaline aqueous isopropanol. Phospholipids, triglycerides and free fatty acids remained in the isopropanol phase. Rockerbie et al. [8] used this method for the analysis of the fatty acid components of cholesteryl esters but Koot-Gronsveld et al. [9] found that the method does not eliminate triglycerides completely from the octane phase and that some isopropanol—fatty acid

TABLE IV

FATTY ACID COMPOSITION OF SERUM CHOLESTERYL ESTER BY TWO DIFFERENT SEPARATION METHODS

Specimen No.	Cholesterol (mg/dl)	Triglycerides (mg/dl)	Fatty acids (%)											
			$C_{16:0}$		$C_{16:1}$		$C_{18:0}$		$C_{18:1}$		$C_{18:2}$		$C_{20:4}$	
			Sep-Pak	TLC	Sep-Pak	TLC	Sep-Pak	TLC	Sep-Pak	TLC	Sep-Pak	TLC	Sep-Pak	TLC
1	251	135	12.2	10.7	3.3	2.7	1.4	1.3	15.0	15.0	55.6	57.9	12.5	12.3
2	271	137	12.3	12.2	9.0	8.5	1.5	1.3	16.0	15.4	50.5	52.0	10.7	10.5
3	172	207	13.3	11.7	4.2	4.5	3.0	0.9	20.7	20.7	52.7	55.5	6.3	6.3
4	213	282	13.2	12.9	5.4	5.2	0.9	0.8	13.0	13.4	54.1	54.3	13.4	13.4
5	296	203	13.0	12.4	4.9	5.2	0.8	0.6	23.1	22.6	49.0	49.6	9.5	9.6
6	266	167	12.9	11.9	4.8	4.6	1.1	1.0	22.0	22.6	49.5	50.0	9.7	9.9
Mean	245	189	12.8	12.1	5.3	5.1	1.5	0.9	18.3	18.3	51.9	53.2	10.4	10.3
S.D.	45	55	0.5	0.8	2.0	1.9	0.8	0.2	4.2	4.2	2.7	3.3	2.5	2.5

esters also contaminate the cholesteryl ester fraction. This was confirmed by Rockerbie et al. [10]. Our method does not present this problem since cholesteryl esters are isolated completely by the Sep-Pak cartridges.

A direct determination of the linoleate to oleate ratio in serum cholesteryl esters has been published by Bernert et al. [11] using reversed-phase high-performance liquid chromatography. The linoleate to oleate ratio tended to have a significantly higher value than that of the GLC method. The reason for this difference has not been clarified. Our Sep-Pak separation of cholesteryl esters may be able to solve this problem by producing the pure fraction of cholesteryl esters.

The silica Sep-Pak cartridge procedure described here offers definite advantages over the traditional TLC separation of cholesteryl esters.

The precision and recovery studies showed that the Sep-Pak method is comparable to the conventional TLC method. There is no statistical significance between these two methods. Although the traditional lipid extraction by chloroform—methanol can be combined with the Sep-Pak method, the *n*-octane lipid extraction offers a slightly better recovery and also the convenience, since the octane layer stays on the top of the mixture. The advantage of speed, convenience and saving of time in sample preparation of the Sep-Pak method provides a rapid and efficient alternative to the TLC method for the separation and analysis of serum cholesteryl esters. The method is simpler, quicker and more readily adaptable than the TLC method.

REFERENCES

1 D.M. Small, N. Engl. J. Med., 297 (1977) 924.
2 G.J. Nelson, in G.J. Nelson (Editor), Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism, Wiley-Interscience, New York, 1971, p. 3.
3 J. Hirsch and E.H. Ahrens, Jr., J. Biol. Chem., 233 (1958) 311.
4 K.J. Kingsbury and D.M.L. Morgan, Biochem. Med., 14 (1975) 209.
5 J. Folch, M. Lees and G.H. Sloane Stanley, J. Biol. Chem., 226 (1957) 497.
6 L.D. Metcalfe and A.A. Schmitz, Anal. Chem., 33 (1961) 363.
7 F. Peter and R.G. Reynolds, J. Chromatogr., 143 (1977) 153.
8 R.A. Rockerbie, R.D. Dobson and J. Frohlich, Clin. Chem., 25 (1979) 1411.
9 E.A.M. Koot-Gronsveld, F.J.M. Schouten, J.T. Knuiman, C.E. West and M.B. Katan, Clin. Chem., 27 (1981) 1148.
10 R.A. Rockerbie, R.D. Dobson and J. Frohlich, Clin. Chem., 27 (1981) 1317.
11 J.T. Bernert, Jr., J.R. Akins and D.T. Miller, Clin. Chem., 28 (1982) 676.