Improved quantitation of plasma lipids by direct gas–liquid chromatography

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ABSTRACT A preliminary digestion of total plasma lipid extracts with phospholipase C, which converts the lysolecithins, lecithins, and sphingomyelins into monoglycerides, diglycerides, and ceramides, respectively, has been shown to facilitate subsequent determination of the plasma lipids by gas–liquid chromatography. A further improvement in the chromatographic elution pattern results from acetylation or trimethylsilylation of the liberated alcohol moieties prior to injection into the chromatograph. If tridecanoin is used as internal standard, quantitative estimates can be rapidly obtained for plasma lysolecithins, free cholesterol, lecithins, sphingomyelins, cholesteryl esters, and triglycerides, as well as for free fatty acids. Other plasma lipids do not occur in sufficiently high concentrations to interfere with the analysis. The determination requires 0.1–0.5 ml of plasma and about 6 hr of processing, but many samples can be processed at a time.

SUPPLEMENTARY KEY WORDS phospholipase C · lysolecithins · monoglycerides · lecithins · diglycerides · sphingomyelins · ceramides · trimethylsilyl ethers · acetates · trifluoroacetates

We recently reported (1) a method for direct gas chromatographic examination of total lipid extracts and noted that its successful application to quantitative routine analyses required extreme care. The difficulty was due to the fact that a quantitative estimate of glycerophosphatides (present in most samples) depended upon their effective pyrolysis in the injector of the gas chromatograph. A more satisfactory dephosphorylation could be brought about by the treatment of these lipids with phospholipase C. This enzyme had earlier been shown to effect complete release of phosphoryl choline from both lecithins and sphingomyelins without attacking the respective glyceride and ceramide end products (2). It remained to be demonstrated that the enzymatic reaction was not inhibited or otherwise interfered with by the other lipids present in the extract, and to show that the digestion products could be accommodated in the over-all elution pattern.

The present report shows that phospholipase *C* from *Clostridium welchii* is satisfactory for the dephosphorylation of lysolecithin, lecithin, and sphingomyelin present in the total lipid extracts of human plasma. Although the enzyme also releases the diglyceride moieties of phosphatidyl ethanolamine when incubated in the presence of lecithin and sphingomyelin (2), this and other glycerophosphatides occur in normal plasma only to a limited extent (3, 4).

MATERIALS

The synthetic neutral lipid and free fatty acid mixtures referred to in this study were described previously (1). The normal plasma samples were obtained from young adults on controlled experimental diets (5). The plasma samples of patients were obtained through the courtesy of Dr. J. A. Little of the Department of Medicine and Dietetics, St. Michael's Hospital, University of Toronto, Toronto, Canada. Samples of organ lipids were obtained from rats killed in the laboratory during the course of other experiments.

Phospholipase C (α-toxin of *Clostridium welchii*) was purchased from the Sigma Chemical Co., St. Louis, Mo. The enzyme was allowed to "autodigest" for about 30 min under the incubation conditions, and the digest was exhaustively extracted with diethyl ether prior to the addition of the substrate. The trimethylsilylating

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; BSA, bis-trimethylsilylacetamide; HMDS, hexamethyldichlorosilane; TMCS, trimethylchlorosilane.

reagents BSA, HMDS, TMCS, and the HMDS–TMCS mixture in pyridine (Tri-Sil) were purchased from Pierce Chemical Co., Rockford, Ill. Other reagents and solvents were of Fisher Certified Reagent grade quality and were tested for lipid contaminants prior to use. All containers were rinsed with chloroform–methanol 2:1 before use.

METHODS

Preparation of Lipid Extracts

Plasma samples were obtained by low speed (2000 g for 20 min) centrifugation of citrated or heparinized whole blood. About 0.1–0.5 ml of the plasma was quantitatively transferred to a 50 ml centrifuge tube, and 1–5 ml of methanol was added. The contents of each tube were mixed and left to stand for 10–15 min in the dark at room temperature. 2–10 ml of chloroform containing 50–200 μg of internal standard (tridecanoin) was added to each tube, and the contents were again mixed and allowed to stand at room temperature. After 20–30 min, 2–10 ml of distilled water was added to each tube and the contents were mixed. The tubes were stoppered with corks, covered with aluminum foil, and centrifuged at 2000 *g* for 40–45 min. The clear lower layer (chloroform phase) was then transferred to another centrifuge tube by means of a capillary siphon and washed twice with water. The washed chloroform layer was evaporated to dryness in the Rotary Evapo-Mix (Buchler Instruments, Inc., Fort Lee, N. J.). The dry residue was dissolved in 25–100 μl of chloroform, and about 1 μl was injected into the gas chromatograph. The rest of the solution was diluted with diethyl ether and digested with phospholipase C as described below.

Hydrolysis with Phospholipase C

The enzymic hydrolyses were performed essentially as described by Renkonen (2). The pooled ether extracts were washed once with 5 ml of distilled water, and the ether was evaporated under nitrogen. The residue was redissolved in 25–100 μl of chloroform, and about 1 μl was injected into the gas chromatograph. The rest of the solution or aliquots thereof were subjected to appropriate chemical transformations and (or) further conventional analyses.

Diazomethylation

In order to convert the free fatty acids into the methyl esters, we treated a chloroform solution of the lipids with a slight excess of freshly distilled diazomethane (6) in cold diethyl ether (about 1 g/100 ml) until a yellow tinge persisted (5 min). The excess of reagents and the solvents were then evaporated off under nitrogen, and the residue was dissolved in 25–100 μl of chloroform.

Acetylation

To convert any compounds with free hydroxyl groups into the acetates, we dissolved the lipid mixture in dry pyridine (0.1 ml) and added an excess of acetic anhydride (0.25 ml). The acetylation was completed by heating the mixture in a tightly closed vial for 1 hr at 80°C. The acetates were recovered as described earlier (7). The petroleum extract of the acetates was evaporated under nitrogen, and the dry residue was dissolved in chloroform.

Trifluoroacetylation

Similarly we converted compounds with free hydroxyl groups into the trifluoroacetates without concomitant alteration of the lipid esters already present. For this purpose we dissolved the lipid mixture in trifluoroacetic anhydride (0.1 ml) and warmed the mixture at 45°C for 15 min in a tightly stoppered screw-cap vial (8). At the end of the reaction the excess reagent was evaporated under nitrogen and the residue was dissolved in chloroform. 1 μl aliquots were injected into the gas chromatograph.

Silylation

Alternatively, we converted any compounds with free hydroxyl groups into the trimethylsilyl ethers, and any compounds with free carboxyl groups into the trimethylsilyl esters (9), as follows. To the dry lipid residue we added either 0.25 ml of Tri-Sil plus BSA in pyridine or 0.1 ml of HMDS, 0.1 ml of TMCS, and 5 drops of dry pyridine. In either case the reagents were left to stand with the lipid for 1 hr at room temperature. About 1 μl of the reaction mixture was injected into the gas chromatograph.

Thin-Layer Chromatography

Separation of the plasma lipids into the various chemical classes by TLC prior to GLC allowed the identification of the source of the peaks and also provided an independent verification of the proportions of the lipid classes in the mixture. For this purpose the original lipid extract or its derived products were applied as bands to thin layers of silica Gel G (20 × 20 cm, 0.25 mm thick layer) and the plates were developed with appropriate solvents (1, 2, 7). Plasma lysolecithin, sphingomyelin, lecithin, and any phosphatidyl ethanolamine were separated in chloroform–methanol–water 65:25:4, in which solvent all the neutral lipids, including the free fatty acids, moved near the solvent front.

The neutral lipids were quantitatively recovered by eluting appropriate portions of the silica gel with chloroform; the phospholipids were eluted with chloroform–methanol–water–acetic acid 50:40:10:1. The eluted

fractions were washed free of silica gel with distilled water. After evaporation of the solvent, the lipid residue was dissolved in a small amount of chloroform and examined in the gas chromatograph as such or after appropriate derivatization. If necessary, further TLC separations were carried out.

Gas Chromatography

GLC of the mixtures of standards and of the original lipid extracts or their derived products was performed on a Beckman GC-4 gas chromatograph with a specially modified on-column injector (1). The entire GLC system was subsequently calibrated with the appropriate mixture of neutral lipids and their acetyl, trifluoroacetyl, and trimethylsilyl ether derivatives, and the response curves or correction factors were calculated. Because these factors varied from column to column and, occasionally, from day to day, a standard run was needed before and after each series of analyses.

Conventional Analyses

Total free fatty acids, total plasma triglycerides, and total plasma cholesterol were determined as previously described (1). Phospholipids were removed on Florisil columns as described by Carroll (10), except that miniature columns (Pasteur pipettes) were employed and the proportions of the solvents were decreased accordingly. The neutral lipids were eluted with diethyl ether or chloroform and the phospholipids with methanol.

Calculations

The absolute concentrations of the original classes of lipids in the total lipid extract were calculated from the peak areas by means of appropriate correction factors. The weight percentages thus obtained were related to the known weight of the internal standard, and these values were expressed as mg/100 ml of plasma. The factors used for the conversion of the weight of the neutral lipid moieties of the phospholipids into the weights of the original lipids were based on the average molecular weight of the monoglyceride (monostearin) and diglyceride (monopalmitomonostearin) or the ceramide (monostearoyl C_{18}-sphingosine), parts of these lipids, as indicated by the proportions of the appropriate peaks in the GLC elution pattern. Alternatively, the correction factors could be calculated from the known average molecular weight of the fatty acids of plasma lysolecithins, lecithins, and sphingomyelins determined in the laboratory or available in the literature (3, 4). The estimates of the various classes of lipids as obtained by the different methods of derivatization were compared on a molar basis, taking into account the nature of the chemical modification involved and the correction factors dictated by the results of analysis of standards.

The accuracy of the final estimates was tested by reconstitution (7) of the fatty acid composition of the original lipid classes from the fatty acid composition (with methyl heptadecanoate as internal standard) of the products of the corresponding enzymatic or chemical modification.

Routine Analysis

For quantitative routine examination of total plasma lipid extracts, the procedure included the following analytical steps described above: preparation of total lipid extract, hydrolysis with phospholipase C, and silylation with Tri-Sil plus BSA. The analyses were done in duplicate, and in each transformation the entire lipid extract of the preceding step was used. The relative error of such determinations varied from 5 to 10% for the phospholipids and from 2 to 5% for the neutral plasma lipids.

RESULTS AND DISCUSSION

The suitability of GLC for the quantitative analysis of complex neutral lipid mixtures has been well established (11, 12) and was not specifically reexamined in this study. In attempting to determine the optimal conditions for the enzymic dephosphorylation of plasma phospholipids and for the accommodation of the released neutral lipid moieties in the over-all GLC elution pattern, however, original observations were required on the GLC behavior of the new derivatives of common neutral plasma lipids and of those neutral lipids not commonly encountered in normal plasma.

Enzymic Dephosphorylation

According to Phillips and Dodge (4) the concentration of total phospholipids of normal subjects is 228–328 μmoles/100 ml of plasma. Of this, 67.4–73.4% is lecithin, 15.2–19.6% sphingomyelin, 3.9–8.5% lysolecithin, 2.4–4.4% phosphatidyl ethanolamine, and 1.2–2.6% phosphatidyl serine. Williams, Kuchmak, and Witter (3) have reported that other phosphatides and lysophosphatides contribute 0.2–1.4% each. For a determination of the *major* phosphatides in normal subjects, therefore, only the first three classes need be considered.

The quantitative conversion of purified lecithins into diglycerides by phospholipase C from *Clostridium welchii* has been demonstrated in several laboratories (2, 13). When enzymic digestion is performed in wet ether (2, 13, 14), the neutral products of the hydrolysis accumulate in the ether phase and do not affect the activity of the enzyme at the interface. Other neutral lipids originally present in the total lipid extract would be expected similarly to dissolve in the ether phase and not to interfere with the action of the enzyme. Sphingo-

myelin has also been reported (2) to be hydrolyzed by phospholipase C from *Clostridium welchii* in wet ether, with the liberation of ceramides. Despite earlier claims to the contrary (15), the lysolecithins are similarly attacked by this enzyme (16). In addition, the lecithin and the sphingomyelin present in the mixture would catalyze the hydrolysis of other phosphatides by phospholipase C (2). On the basis of these reports, one would expect a nearly complete hydrolysis of all major plasma phospholipids by this phospholipase. The phospholipase C from *Bacillus cereus* might possibly have been a better choice on account of its wider specificity (17), but this enzyme is not readily available.

Table 1 compares the composition of the fatty acids of plasma lysolecithin, lecithin, and sphingomyelin with that of the corresponding glycerides and ceramides liberated by phospholipase C during incubation with total plasma lipids. There is an excellent qualitative and quantitative correspondence among the fatty acid compositions of the original phospholipids and the derived neutral lipids. In all cases the fatty acid composition of these phosphatides agrees closely with that given for normal plasma by Williams et al. (3) and by Phillips and Dodge (4).

Only lecithins and sphingomyelins, however, were completely hydrolyzed and recovered in the respective diglyceride (99%) and ceramide (93%) forms. The lysolecithins were transformed into monoglycerides to the extent of 70–80%. The recoveries are based on GLC of the component fatty acids of the original phospholipids and the derived glycerides and ceramides in the presence of methyl heptadecanoate. The relative error of these estimates was 2–5%. Prior to transmethylation, the various lipid classes were resolved by TLC. The recoveries have not been corrected for minor losses during the manipulation.

GLC of Phospholipids

The improvement in the gas chromatographic properties of the phospholipids as a result of the prior enzymic digestion is illustrated by the elution sequences represented in Fig. 1. The pattern in Fig. 1A was obtained for total plasma phospholipids. These were isolated by TLC from 1 ml of plasma and, after dilution with tridecanoin (200 μg) were injected directly into the gas chromatograph under the usual working conditions. In the elution patterns the major peaks have been designated according to the total number of acyl carbons in the triglycerides having the same retention temperature. In Fig. 1A we see, in addition to the peak for tridecanoin, peaks which result from the pyrolysis of the lecithins and sphingomyelins (carbon numbers 24–26 and 33–42) as well as traces of degradation products of lysolecithins (carbon numbers 20–22). Although the

TABLE 1 FATTY ACID COMPOSITION OF PLASMA LECITHINS AND SPHINGOMYELINS AND OF THE DERIVED GLYCERIDE AND CERAMIDE ACETATES

Fatty Acids	Lipid Classes					
	Lecithins	Diglycerides	Sphingomyelins	Ceramides	Lysolecithins	Monoglycerides*
				moles %		
14:0	tr.	tr.	tr.	tr.	tr.	2.0
16:0	29.6	31.2	30.4	29.8	40.6	34.9
16:1	0.8	0.5			2.4	2.1
17:0	1.3	0.9	1.5	2.0	0.5	1.0
18:0	13.1	12.9	11.3	11.0	27.0	30.7
18:1	10.8	11.5	2.8	3.4	13.2	15.4
18:2	29.6	29.9	2.9	3.2	15.1	13.8
20:0	0.2	0.3	3.3	3.8		
20:2	0.4	0.5	1.2	0.9		
20:3	2.6	2.5	0.3			
20:4	6.5	5.2				
22:0			7.1	7.2		
22:1			13.6	10.2	1.2	tr.
23:0			3.0	4.0		
24:0			3.8	4.8		
24:1			18.0	18.7		
22:5	0.7	0.6				
22:6	3.6	3.3				

* The monoglycerides corresponding to the lysolecithins of this preparation were not separately recovered. The fatty acid composition given in the table was determined for a different lysolecithin preparation from which the monoglycerides were isolated.

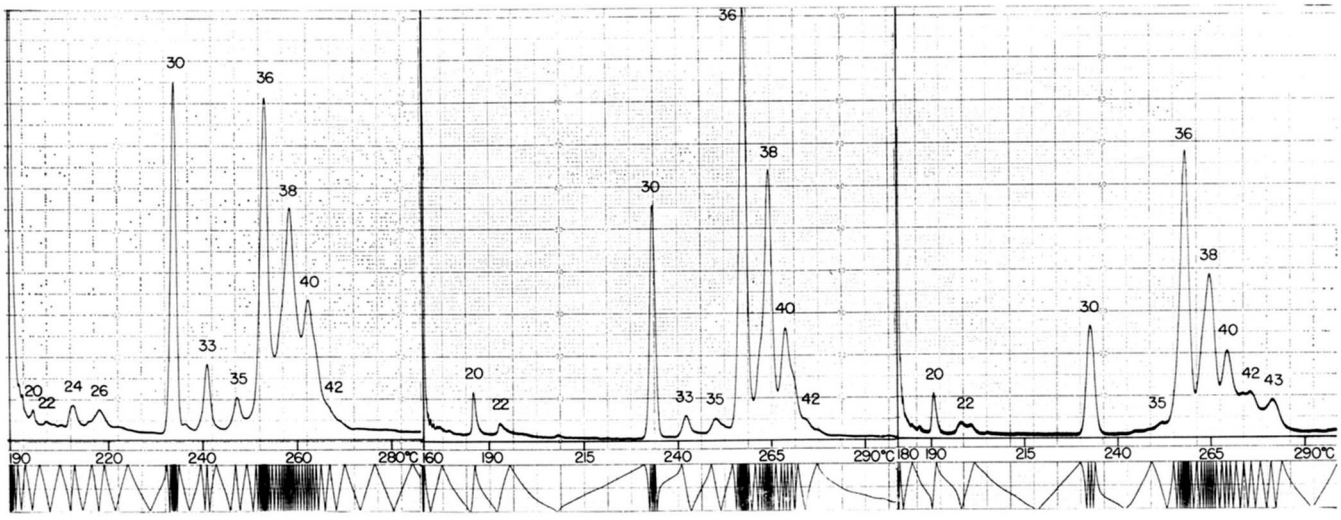

FIG. 1. GLC of mixed phosphatides of human plasma. A, direct GLC; B, GLC after hydrolysis with phospholipase C; C, GLC of acetylated hydrolysis products. Instrument: Beckman GC-4 gas chromatograph with specially modified on-column injector (1). Column: 45 cm × 3 mm O.D. stainless steel tubes packed with 3% JXR on 100–120 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, Pa.). Carrier gas: N_2, 150 ml/min. Injector, 300°C. Hydrogen flame detector, 350°C. Temperature program: as given in the figure.

Peaks identified by total number of acyl carbon atoms. 20–22, monoglycerides or their diacetates; 24–26, unknown pyrolysis products; 30, tridecanoin (internal standard); 33–43, pyrolysis products of lecithins and sphingomyelins, or mixed diglycerides and ceramides (or their acetates).

peaks are fairly symmetrical, there are signs of incomplete equilibration and resolution. Furthermore, the yield of the pyrolysis products may be as low as 60–70% of the theoretical values (1), and each lecithin species may produce a pair of peaks. The elution pattern in Fig. 1B was obtained with the same phospholipid preparation after digestion with phospholipase C. In addition to the improved peak shape and resolution, the recovery of neutral lipids derived from the phospholipid molecules was increased. The higher recovery (an average of 5% over pyrolysis) is largely due to the diglycerides released from the lecithins. The monoglycerides and the ceramides, each of which contain two free hydroxyl groups, show considerable tailing and are still poorly recovered. The pyrolysis peaks 24 and 26, however, are no longer apparent. The tailing is further reduced in Fig. 1C, which shows the elution pattern obtained for the above enzyme digest after acetylation. Compared with the internal standard, the area due to the neutral lipid parts of the phospholipids has now been increased an average of 14%. The monoglycerides containing one C_{18} fatty acid (peak 22) are resolved on the basis of their shape, with the unsaturates emerging ahead of the saturates. As a result of acetylation the retention temperature of the ceramides has been increased by an amount corresponding to about two methylene units, which gives a new peak with carbon number 43. For the same reason, peaks 33 and 35 are shifted to the right. However, a new peak of carbon number 33 representing other, lower molecular weight species may appear (see Fig. 3 and 4). Acetylation of the diglycerides resulted in no significant change in their retention time (11, 18, 19).

Fig. 2 shows the GLC elution patterns recorded separately for the acetates of diglycerides and ceramides as well as for the trimethylsilyl ethers of ceramides. Prior to GLC the two acetate classes were separated by TLC. The ethers were made by silylation of the original ceramides. In Fig. 2A we see the usual pattern of the diglyceride moieties of plasma lecithins (13). The ceramide acetates constitute a complex mixture, with peaks corresponding to both odd and even carbon numbers. Although plasma sphingomyelins are known (14) to contain odd and even carbon number fatty acids, they do not occur in proportions which can entirely account for this pattern. Furthermore, after hydrogenation the ceramide acetates yield GLC peaks similar to those given for the trimethylsilyl ethers in Fig. 2C. The latter derivatives have been shown (14) to be suitable for GLC of plasma ceramides by mass spectrometry. The elution temperatures of the trimethylsilyl ethers of the ceramides are slightly higher than those of the hydrogenated ceramide acetates of corresponding carbon number. The GLC patterns of the original and the reduced ceramide acetates are best explained by assuming that a pair of peaks is obtained for each carbon number. This interpretation would be compatible with a partial loss of the allylic acetyl groups during hydrogenation (20) and upon evaporation in the gas chromatograph.

The apparent irregularities in the GLC behavior of the ceramide acetates did not lead to any difficulty in the

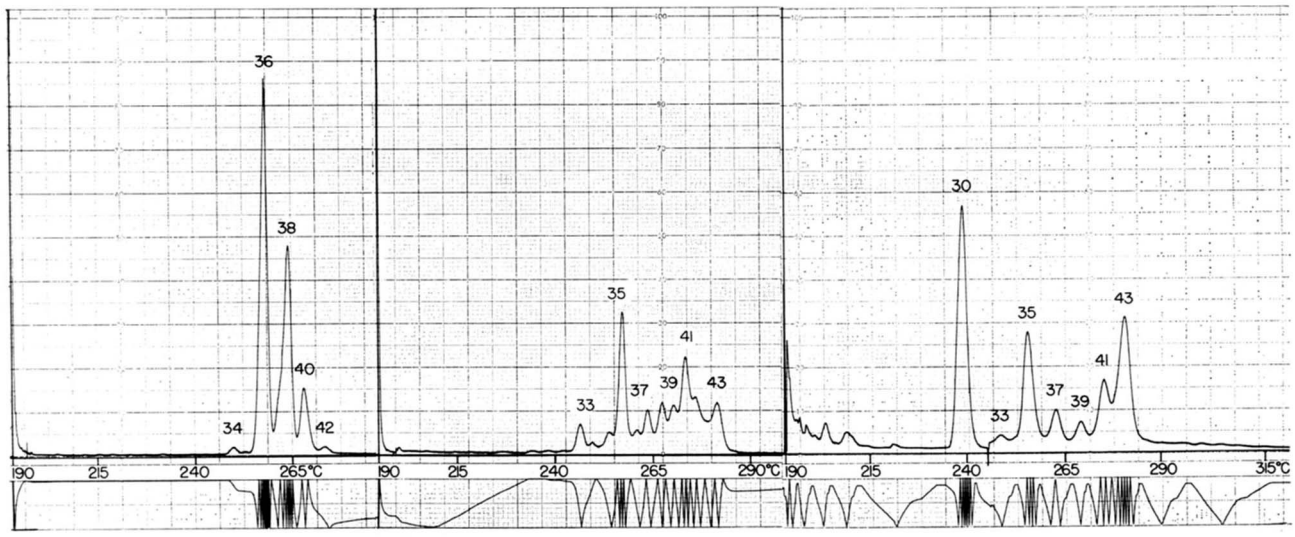

FIG. 2. GLC of diglyceride and ceramide acetates derived from plasma lipids. A, diglyceride acetates; B, ceramide acetates; C, trimethylsilyl ethers of ceramides plus tridecanoin (30). Instrument and operating conditions as in Fig. 1. Other peaks as in Fig. 1.

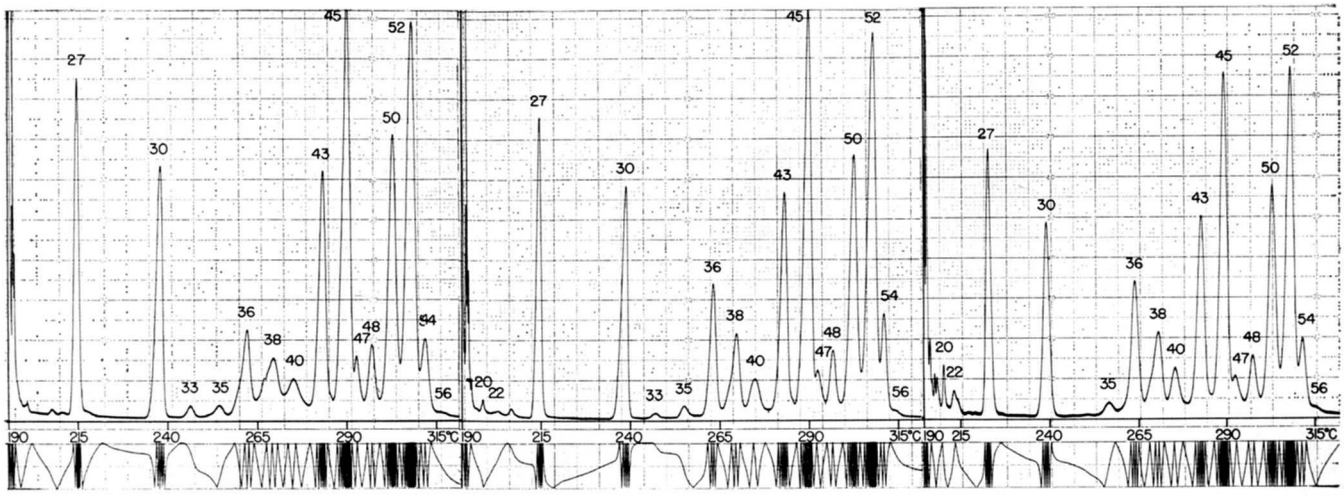

FIG. 3. GLC of plasma lipids. A, direct GLC; B, GLC of plasma lipids after hydrolysis with phospholipase C; C, GLC of acetylated hydrolysis products. Instrument and operating conditions as in Fig. 1. Peak 27, cholesterol; peaks 30–43 as given in Fig. 1; peaks 43–47-cholesteryl esters; higher peaks, triglycerides.

quantitation of the ceramides. They consistently contributed 15–18% of the area represented by the diglycerides and ceramides. This proportion corresponded well to that obtained for the trimethylsilyl ethers of the ceramides as well as for the spingomyelins in the total plasma phosphatides (3, 4).

GLC of Total Plasma Lipids

Fig. 3 shows the improvement in peak resolution and in the recovery of the total plasma lipids resulting from a digestion of the lipid extract with phospholipase C prior to GLC. The sample selected for this illustration was obtained from a young adult subsisting on a fat-free diet, which apparently had led to a considerable increase in the fasting plasma triglycerides. The GLC pattern obtained without enzyme treatment is shown in Fig. 3A. The patterns shown in Figs. 3B and 3C indicate, in relation to the internal standard, a progressive increase in the areas contributed by phospholipids as a result of enzymic digestion and both enzymic digestion and acetylation, respectively.

Table 2 gives estimates of the various neutral lipid ester classes on repeated injection of a sample and for a duplicate analysis of another sample. The relative error for most components is less than ±5%, and the GLC estimates are in rather good agreement (±10%) with the few conventional analyses available for these plasmas (total phosphorus, cholesterol, and triglycerides).

Despite the obvious success with acetylation, we felt that this method of analysis could possibly be further

TABLE 2 REPRODUCIBILITY OF REPEAT DETERMINATIONS OF VARIOUS PLASMA LIPIDS BY GAS CHROMATOGRAPHY

Lipid Component	Repeat Injection of Acetates*			Parallel Analyses of Acetates†		
	A‡	B‡	C‡	D§	E§	F§
		μmoles/100 ml‖				
Lysolecithins	9.7 ± 1.3	8.3 ± 0.6	21.3 ± 2.1			
Free cholesterol	154.7 ± 3.7	90.3 ± 0.6	121.0 ± 4.3	255, 266	351, 372	223, 215
Lecithins + sphingomyelins	153.0 ± 4.1	160.3 ± 3.7	204.3 ± 6.7	269, 279	520, 585	229, 214
Cholesteryl esters	454.7 ± 7.5	337.3 ± 10.3	370.3 ± 16.2	803, 803	696, 698	356, 380
Triglycerides	127.7 ± 10.3	196.7 ± 3.7	72.0 ± 2.9	115, 111	1540, 1550	1240, 1270

* Three injections into gas chromatograph; mean ±SD.
† Two complete parallel determinations.
‡ Normal young adults on free-choice diets.
§ Patients with disorders of lipid metabolism.
‖ The number of moles of each lipid class was obtained by dividing the weights of the appropriate neutral lipid classes by 444 (mol wt of diacetyl ester of monostearin), 429 (mol wt of the acetyl ester of cholesterol), 639 (mol wt of the monoacetyl ester of monopalmito-monostearin), 651 (mol wt of cholesteryl oleate), and 835 (mol wt of dipalmito-monostearin.)
Conventional analyses (as μmoles/100 ml plasma). Total cholesterol: sample A, 550; sample B, 427; sample C, 480; sample D, 990; sample E, 1000; sample F, 605. Triglycerides: sample D, 130; sample E, 1300; sample F, 1100. Lipid-soluble phosphorus: sample D, 300; sample F, 250.

improved by substituting trimethylsilylation or trifluoroacetylation for the acetylation step. Acetylation requires heating of the sample for complete reaction and subsequent removal of the reagents by partitioning with water and organic solvents. Although satisfactory elution patterns can be obtained after injection of the entire reaction mixture, the lifetime of the column is seriously shortened. Trifluoroacetylation and trimethylsilylation, on the other hand, may be accomplished at room temperature or by gentle heating, and the reagents are readily removed by evaporation. Furthermore, silylation of the enzyme digest of the total lipid extract also leads to the formation of the trimethylsilyl esters of free fatty acids, which can then be determined together with the other plasma lipid classes. This obviates the need for diazomethylation of the lipid extract (1). The suitability of the silyl esters of fatty acids for GLC analysis has been investigated in several laboratories (12, 21), and the results agree satisfactorily with those obtained by analysis of methyl esters. Finally, trifluoroacetylation could be an advantage in those cases where there was danger of overlapping between the long-chain ceramides and cholesteryl plamitate, since the trifluoroacetates have considerably shorter retention times than either the silyl ethers or the acetates of the partial glycerides and ceramides.

Fig. 4 compares the elution patterns obtained for the acetates, trimethylsilyl ethers, and trifluoroacetates prepared from the same enzyme digest of total plasma lipids. The acetates give the most compact peaks for the diglycerides and ceramides and allow the most sensitive detection. The silyl ethers and trifluoroacetates give less

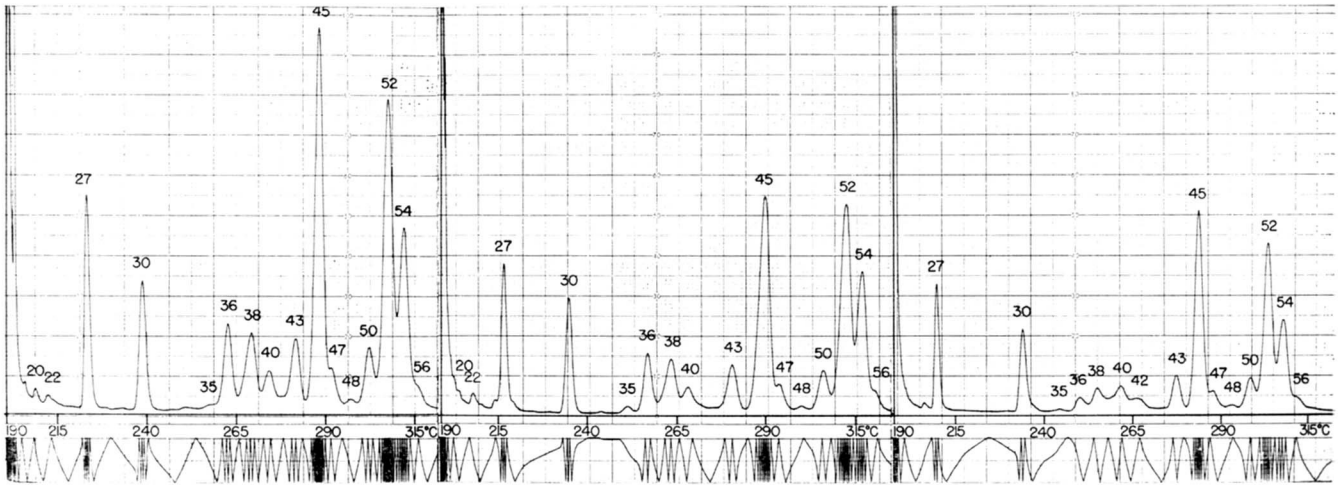

Fig. 4. GLC of plasma lipids after enzyme hydrolysis and derivatization. A, acetates; B, trimethylsilyl ethers; C, trifluoroacetates. Instrument and operating conditions as in Fig. 1. Peak identities as in Fig. 3.

TABLE 3 DETERMINATION OF THE LIPID COMPOSITION* OF PLASMA FROM FASTING NORMAL SUBJECTS ON FREE-CHOICE DIETS

Lipid Component	Males (n = 7)	Females (n = 6)
	μmoles/100 *ml*†	
Lysolecithins	16.9 ± 2.8	12.2 ± 2.0
Free cholesterol	117 ± 11.7	119 ± 12.9
Lecithins + sphingomyelins	139 ± 23.2	143 ± 14.0
Cholesteryl esters	305 ± 46.7	325 ± 104.7
Triglycerides	94.0 ± 34.6	62.2 ± 23.0

* Values are means ± SD from 13 plasma samples, estimated after enzymatic digestion and silylation.

† The number of moles of each lipid class was obtained by dividing the weights of the appropriate neutral lipid classes by 504 (mol wt of the ditrimethylsilyl ether of monostearin), 460 (mol wt of the trimethylsilyl ether of cholesterol), 670 (mol wt of the trimethylsilyl ether of monopalmito-monostearin), 651 (mol wt of cholesterol oleate), and 835 (mol wt of dipalmito-monostearin).

discrete peaks and impair the recognition of the underlying distribution of the molecular weights of the components. There was also little improvement in the elution pattern of the monoglycerides as a result of silylation. The elution of the silyl esters of the fatty acids (not shown in Fig. 4) compared favorably with that noted previously (1) for the methyl esters prepared by diazomethylation of total plasma lipids. Relative to the acetates, the combined recoveries of the diglycerides and ceramides as the silyl ethers were 103% and as the trifluoroacetates 87%. The relative error was 2–5%. The low recoveries of the trifluoroacetates were due to the instability of these derivatives at the elevated temperatures necessary for successful GLC of the triglycerides. The shorter time required for derivatization and the more satisfactory elution of the ceramides (see Fig. 2) make the silyl ethers, therefore, the derivatives of choice in routine analyses.

Table 3 gives the results of a series of determinations of plasma lipids in young adults on free-choice diets. Although not previously expressed in this form (μmoles/100 ml plasma), the estimates for the various lipid classes are in the normal range (1, 3, 4), and agree rather closely (±10%) with the values obtained by conventional analyses for total lipid phosphorus, cholesterol, and triglycerides.

Altogether, some 400 different plasma samples have now been examined by this method; the resultant data correlate well with the clinically determined healthy or diseased states. Apart from a rapid determination of the various lipid classes, this method provides a plasma lipid pattern which apparently varies both with the diet and with the metabolic state of the individual. The method surpasses conventional determinations not only in speed and accuracy but also in the amount of accessory information obtained concerning composition within lipid classes. The method is susceptible to analysis of ^{14}C-labeled lipids, provided suitable GLC systems are available, as well as to eventual automation.

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