use of such columns may no longer be necessary nor desirable. For these reasons we believe that phases of moderate to medium polarity among the stable silicone polymers, such as SE 52 and XE 60, will prove most useful in conjunction with the present method. Stability in both JXR and SE 52 was not the least contributing factor in promoting favorable conditions for analyses at high instrumental sensitivity. These phases were the most stable among all types available in the nonpolar (JXR) and the more polar (SE 52) categories. Dimethylpolysiloxane JXR is somewhat more stable than SE 30, although similar to SE 30 in general characteristics.

Newly packed columns had a tendency to become more porous with concomitant decrease in retention time. Original retention times could be maintained by the routine adjustment of helium flow using a compound of average retention time as reference. Calibration curves then remained valid for prolonged periods. The rapidity and reliability of calibrating operations by the proposed method simplified the routine checking of column characteristics and permitted the determination of analytical variance (1,2). The last feature alone is of considerable import in that it gives access to an estimation of the significance of relatively small analytical differences, i.e., to a clearer interpretation of results.

The principle at the basis of the factors listed in Table IV is identical to that applied by Knights and Thomas (9) in their determination of ΔR_{Mg} factors. Both series of factors represent specific structural contributions to the relative retention time, i.e., Rt'_R in the present nomenclature and r for the above authors. The factors in Table IV are dimensionally different however, since the columns, conditions, and reference compounds were different. Both types are closely related to the steroid numbers, SN, and F factors of VandenHeuvel and Horning (10) since the latter factors are also related to relative retention times by formally simple expressions. Thus

$$SN_X = 19 + \Sigma F = 19 + 8 \log Rt'_{RX}/\log Rt'_{RC}$$

where Rt'_{RX} and Rt'_{RC} are the relative retention times for a compound X and cholestane, respectively, and both relative to androstane, gives the value of a steroid number. It is evident from this expression that the range of validity of SN_X will correspond to a range of conditions where relative retention times Rt'_R are sufficiently constant, as the proponent authors have pointed out (10).

The relation

$$SN_X = 19 + 8 \frac{\log Rt'_{RX} - \log Rt'_{RA}}{\log Rt'_{RC} - \log Rt'_{RA}}$$

where all Rt'_R values are relative to the same reference compound, and Rt'_{RA} corresponds to androstane, allows the calculation of SN_X values from Rt_R data obtained with any column. It would seem that SN factors represent a more involved expression of the relative retention times than the ΔR_{Mg} factors of Knights and Thomas and those derived in the present study. The latter are valid from 175C to 200C with JXR columns.

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Ultramicro Fatty Acid Analysis of Polar Lipids: Gas-Liquid Chromatography After Column and Thin-Layer Chromatographic Separation

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Abstract

A procedure is described for the analysis of the fatty acid composition of polar lipid classes in the nanogram range. The lipids are first fractionated by column chromatography followed by further separation into pure lipid classes by thin-layer chromatography. Lipid spots scraped from the thin-layer plates are esterified directly (i.e., without prior elution) with 6% sulfuric acid in methanol. The methyl esters are then analyzed by gas-liquid chromatography with a hydrogen flame ionization detector. Samples of 200 nanograms or less give accurate results with helium as carrier gas, oxygen rather than air to support combustion, careful adjustment of the recorder and general attention to optimum electrical connections, dissociation of the column oven from the recorder and electrometer, and careful preconditioning of columns. Under proper conditions the base line is stable and a 10% of full scale deflection of the recorder can be obtained from 1 nanogram of a methyl ester, allowing highly precise analyses of fatty acid composition from the amount of lipid obtainable from one spot on a thin-layer chromatogram. Control studies demonstrated that extraneous peaks did not arise from the procedure or from the sphingosine and dihydrosphingosine of sphingolipids. The thin-layer chromatographic procedure did not influence the fatty acid composition of a pure sample of glucocerebroside isolated by column chromatography and the method was applied to lecithin and sphingomyelin or normal and pathological human brain specimens.

Introduction

DETERMINATION OF FATTY ACID composition of specific lipid classes is a difficult problem for investigators working with very small samples. The preferred general procedure is gas-liquid chromatography

(GLC) on both polar and nonpolar liquid phases, at several different concns and at several different temps in order to accurately detect and quantitate all of the components. It is also frequently desirable to hydrogenate the esters, particularly as an aid in identifying polyenoic acids. The occurrence of miscellaneous components in the chromatogram (such as reaction artifacts, branched-chain acids, hydrocarbons, acetals, alcohols, etc.) can cause confusion (1) and requires additional procedures to eliminate their interference.

The extremely sensitive ionization detectors for GLC minimize the amt of sample needed for a complete fatty acid analysis. The purpose of this report is to describe a means whereby the practical working level of a typical GLC system can be extended to the nanogram level and to illustrate the application of this extreme sensitivity to the analysis of fatty acids from human spleen and brain lipids obtained by column chromatography and thin-layer chromatography (TLC).

Materials and Methods

Gas Chromatographic Apparatus. The instrument used in these studies was an Aerograph Model A-600C equipped with a hydrogen flame ionization detector. Our previous experience with this system (2) showed it to be linear in its response at extremely high sensitivity both with and without a stream splitter. In addition to its rapid response, the flame detector is insensitive to water. This is particularly desirable when it is impractical to dry samples over a suitable desiccant.

GLC Columns. Five percent (w/w) SE-30 coated on 60/80 mesh Chromosorb W (Wilkens Instrument and Research, Inc.) packed into a 5 ft × ⅛ in. stainless steel column was used as the nonpolar liquid phase. Columns were conditioned at 250C for 48 hr before being used for analysis and operated at either 210C or 225C, the latter temp as a check for long chain esters whose peaks are flattened at the lower temp.

The polar liquid phase was 10% (w/w) ECNSS-S coated on 100/120 mesh Gas Chrom P (Applied Science Laboratories) and packed into a 5 ft × ⅛ in. stainless steel column. This liquid phase is an organosilicon polyester capable of producing excellent separation of saturated and unsaturated fatty acid methyl esters (3). It is stable below 225C and has a useful life of 3–6 months depending upon the operating temp and the polarity of the carrier solvent. ECNSS-S columns were conditioned at 210C for 24 hr and then operated at either 187C or 205C.

Carrier Gas. Helium was used as carrier gas with both columns. Optimal flow rates were found to be 26 ml/min at 12 psig with the nonpolar phase and 3 ml/min at 14 psig with the polar phase. The slow flow rate with the polar phase resulted in a slight loss of sensitivity. This was compensated for by operating at max input impedance attenuated 4x as compared to an 8x attenuation with the nonpolar phase.

Flame Gases. Hydrogen and air are the usual mixtures for the flame ionization detector. However, we found that the high flow rate (400 ml/min) of the air, which supports combustion, produces a cooling of the flame tip leading to thermionic noise and a loss of sensitivity. This was alleviated by substituting pure oxygen for air resulting in a hotter flame and at least a three-fold increase in sensitivity without any appreciable base line noise. Optimal gas flow rates were found to be 20 ml/min of hydrogen and 200 ml/min of oxygen. Under these conditions 0.001 μg. of methyl stearate elicited a recorder response of approx 10% of full scale.

It is advisable to use constrictors in the oxygen and hydrogen lines before they enter the flame base. Their use permits high head pressure resulting in a more accurate control of flow rates than can otherwise be achieved. Constrictors can be fashioned from 0.01 or 0.02 in. (ID) capillary bore stainless steel tubing of 12–18 in. in length.

CAUTION: The mixture of hydrogen and oxygen described above is highly explosive unless adequate safety precautions are taken. The flame should never be ignited unless the detector is totally vented to the atmosphere. This can be conveniently accomplished by removing the cap from the detector cylinder. Accumulated gases can be flushed by blowing them out and the flame can then be safely ignited. The gas chromatograph should be located in an adequately ventilated room, preferably with an exhaust fan vented to the outside. As a further precaution, smoking and the use of open flames in the same room should be strictly prohibited.

Sample Volumes and Quantitation. The total weight of the samples of lipid classes analyzed was 400 to 1000 μg, although much smaller samples could have been used. The esters were made to 1.0 ml with hexane and a 0.5–1.0 μl sample was injected into the gas chromatograph for analysis. This provided a level of approximately 0.2–1.0 μg of total esters for each GLC analysis.

Quantitation was achieved by calculating relative percent composition from the products of adjusted retention time and peak height. Each individual product was divided by the sum of the products to give percent composition. Calibration of the instrument with mixtures of pure methyl esters obtained from the National Heart Institute showed a relative error of less than 2% with all components by this procedure. At extreme sensitivity as well as at lower levels, calibration with pure standards of known composition is mandatory for accurate quantitation (1).

Hexane (redistilled) is the most generally useful solvent for sample injection. Carbon disulfide offers the advantage, however, that it does not produce a response with the flame ionization detector (4). In the present study we were able to use this solvent to great advantage with the SE-30 liquid phase, but its use with ECNSS-S shortened the life of the column and led to the occurrence of spurious peaks in the chroma-

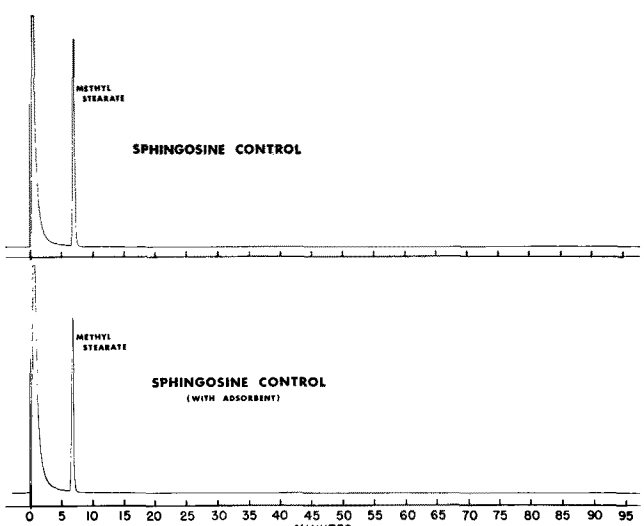

FIG. 1. Gas chromatographic elution patterns for second control series on the polar liquid phase.

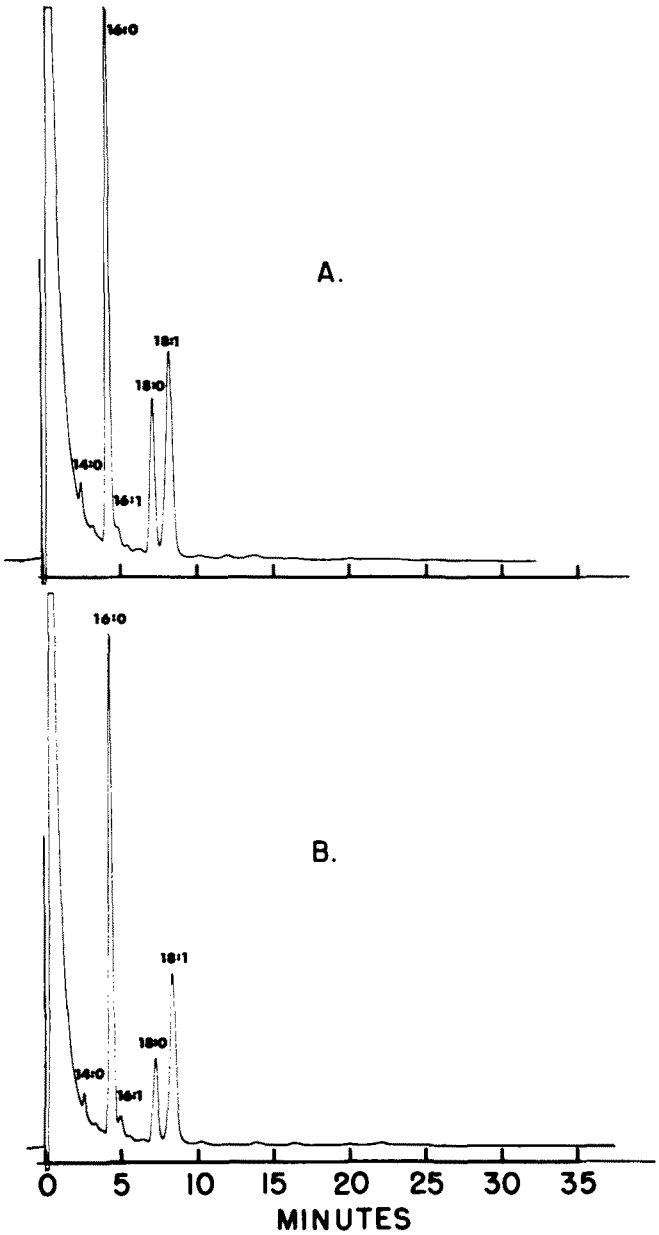

FIG. 2. Typical gas chromatographic elution patterns of the methyl esters of fatty acids from human brain lecithin obtained by direct methylation of the adsorbed lipid after TLC. Chromatogram A is from Gaucher's disease and B from Tay-Sachs disease. Chromatographed on 10% ECNSS-S at 187C.

tograms. Steam cleaning the column by periodically injecting several 5 μl portions of distilled water eliminates the artifacts, but does not prevent the shortened life of the column. Regardless of the choice of solvent, redistillation before its use is mandatory.

Lipid Samples. Lecithin and sphingomyelin from normal and pathological human brains and a glucocerebroside sample isolated from the spleen of a patient with Gaucher's disease were analyzed.

Lecithin and sphingomyelin were eluted from a DEAE cellulose column as a single fraction (mixed with cerebrosides) and then separated by TLC with a mixture of silicic acid/magnesium silicate (9:1, w/w) as adsorbent and chloroform/methanol/water (65/25/4, v/v) as developing solvent (5). The lipids were located by spraying with 0.001% aqueous Rhodamine 6G and viewing the damp plates under UV light. The spots were scraped from the plate with a razor blade while the adsorbent was still moist, quantitatively transferred to a test tube and dried in a desiccator under a nitrogen atmosphere.

Methyl esters were prepared by direct transesterification without prior elution of the lipids. The reaction was carried out with 2 ml of 6% (v/v) methanolic sulfuric acid in a nitrogen atmosphere and in a sealed tube at 110C overnight with adsorbent and lipid from two TLC plates (20 × 20 cm). On the following day the tubes were cooled, opened, and 1 ml of water was added. The esters were then extracted with several 0.5 ml portions of hexane and analyzed by GLC.

Identification of Peaks. Identification of the fatty acids was made by carbon number determined by the procedure of Woodford and van Gent (6). The identification of the unsaturated acids was checked further by hydrogenation over metallic palladium (7) followed by GLC on both phases.

Results and Discussion

Base Line Stability. Figures 1–3 illustrate the base line stability obtained under the present conditions of extreme sensitivity as well as the absence of artifactual components from the TLC adsorbent or the methylation reaction. A stable base line is necessary for analysis at extreme sensitivity. Careful attention to the instrument's electrical system aids in reducing excessive noise. In addition to determining that all electrical connections are tight, it is desirable to electrically dissociate the recorder from the electrometer and column oven to avoid interference (4).

Proper adjustment of recorder gain and damping is vital to base line stability and accurate quantitation. Unfortunately, this aspect of instrument adjustment is frequently overlooked and is seldom discussed. An overdamped recorder gives a diminished pen response giving rise to low results for early components. Underdamping causes overrunning of the pen which leads to high results with early peaks. Optimal damping is achieved by running the pen to 80% of full scale by means of the balance current of the electrometer. The instrument is then attenuated to half of that value and damping adjusted until the pen returns precisely to 80% without overriding this point or creeping up to it when the original setting is restored.

Excessive recorder gain produces a noisy base line whereas insufficient gain reduces the recorder response speed and may cause the base line to assume a stair-step appearance. Proper adjustment is achieved at a point that is best described as "incipient noise."

Experimental Controls. One control series was designed to establish whether or not the presence of the TLC adsorbent had any adverse effects on the reaction system when compared with conventional procedures. For this purpose a sample of pure glucocerebroside was used. The sample was examined before and after

TABLE I

Comparison of the Fatty Acid Composition of Pure Glucocerebroside Before and After Thin-Layer Chromatography Followed by Direct Methylation of the Adsorbed Lipid

Methyl ester	Percent composition		
	Cerebroside	Cerebroside + adsorbent [a]	Cerebroside after TLC
16:0	22.51	21.03	21.44
17:0	0.85	1.06	0.99
18:0	5.70	5.33	5.43
19:0	0.50	0.62	0.50
20:0	3.53	3.50	3.60
21:0	0.42	0.44	0.47
22:0	20.26	19.47	20.50
23:0	11.39	10.60	11.37
24:0	18.01	18.65	18.71
24:1	16.84	19.29	16.99
	100.01	99.99	100.00

[a] Adsorbent added to the pure cerebroside and the mixture then transesterified at 110C

TLC. The analysis of the glucocerebroside controls is summarized in Table 1. The results obtained with the pure cerebroside without TLC were identical to those obtained with the same preparation after TLC. There was no evidence of extraneous substances from either the adsorbent or the reaction procedure. Transesterification was carried out at 70C in the absence of adsorbent, but in the presence of adsorbent a higher temp (110C) is needed to obtain complete reaction.

A second control series was run to determine whether extraneous material arises from either the reaction or the adsorbent, and whether sphingosine from sphingolipids will appear in the hexane solution and give rise to a peak on the chromatogram. An equimolar mixture of stearic acid and sphingosine (containing some dihydrosphingosine) was methylated in the presence and absence of adsorbent to check this point. Only one peak, that from methyl stearate, was obtained (Fig. 1). There was no evidence of interference from the sphingosine or dihydrosphingosine nor did extraneous material arise from either the reaction or the adsorbent.

These findings demonstrate that the prior elution of the lipid as described by other investigators (8–10) is an unnecessary and time-consuming step. In addition, direct esterification of the adsorbed lipid eliminates the possibility of alteration (e.g., from autoxidation) as a result of the additional handling required with an elution procedure. There is no possibility of loss of any of the components with direct esterification of the adsorbed lipid and the resulting fatty acid analysis appears to be a true reflection of the composition of the total lipid class.

The use of methanolic sulfuric acid for this procedure is highly desirable in view of a recent report by Johnston and Roots (11) describing contamination from the use of methanolic hydrochloric acid as a methylating reagent in the ultramicro analysis of methyl esters by GLC. Inasmuch as our analytical conditions are designed for sample sizes of approx 1/20 the levels that they reported, the necessity of avoiding contamination is extremely important. Lindgren et al. (12) discussed sources of contamination other than methylating reagents. Strict observance of these precautions makes possible ultramicro GLC for routine practical analyses of fatty acids with very small samples (such as individual lipid spots obtained by two-dimensional TLC in which the amount of lipid from one spot may be as low as 0.5 μg).

Brain Lipids. Fatty acid composition of human brain lecithin is very simple. Figure 2 shows typical chromatograms obtained from pathologic brains and illustrates this simplicity. Of particular interest was the absence of polyenoic acids even when the samples were chromatographed at very high load levels.

Figure 3 shows typical traces from brain sphingomyelins. The chromatograms illustrate the strikingly different fatty acid composition of this lipid class compared to lecithin. Brain sphingomyelin is very rich in stearate and also contains large amounts of lignocerate and nervonate. These results for whole brain are also in agreement with the findings of Bernhard and Lesch (13).

General Applicability of the Method. The present method should be widely applicable. The combination of DEAE column chromatography and TLC is advantageous since the two lipid classes most susceptible to autoxidation, phosphatidyl ethanolamine and phosphatidyl serine, are recovered in pure form from the column and thus do not require exposure to air through additional manipulations. The great sensitivity of the GLC method makes possible the analysis of a few micrograms of a lipid class obtained from one spot on a two-dimensional chromatogram. Some changes from exposure to air must be expected with both phosphatidyl ethanolamine and phosphatidyl serine if two-dimensional TLC (without column chromatography) is used for their isolation unless special care is taken and the operations are done largely under a nitrogen atmosphere.

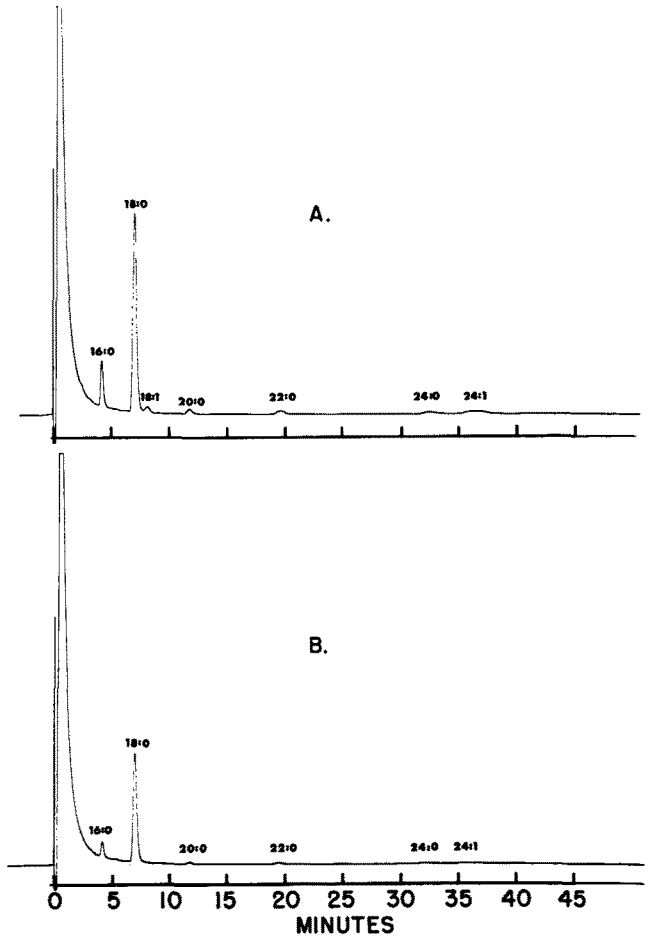

FIG. 3. Typical gas chromatographic elution pattern of the methyl esters of fatty acids from human brain sphingomyelins obtained by direct methylation of the adsorbed lipid after TLC. Chromatogram A is from a normal adult brain, B from Tay-Sachs disease.

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