Improved reagent for trimethylsilylation of sphingolipid bases

H. E. CARTER and R. C. GAVER

Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana, Illinois 61801

ABSTRACT This paper describes the trimethylsilylation of sphingolipid bases under conditions that give derivatives of improved stability. The retention times of the common C_{18} and C_{20} long-chain bases, including the anhydro bases, obtained on a commercially available gas–liquid chromatographic column with a nonpolar stationary phase are given. Data are also presented on the separation of the *erythro* and *threo* isomers of sphingosine and dihydrosphingosine, as the trimethylsilyl ethers of the *N*-acetyl derivatives.

KEY WORDS sphingolipid bases · gas–liquid chromatography · trimethylsilyl ether derivatives · separation · long-chain bases · *erythro* and *threo* isomers · trimethylsilylation · *N*-acetyl bases · C_{18}- and C_{20}-sphingosine, dihydrosphingosine, and phytosphingosine · dehydrophytosphingosine · anhydro-C_{18}-phytosphingosine · anhydro-C_{18}-dehydrophytosphingosine

THE ANALYSIS of various long-chain bases (LCB) by gas–liquid chromatography of the trimethylsilyl ether (TMSi) derivatives has been reported. Gaver and Sweeley (1), using the trimethylsilylation reagent originally developed for carbohydrates (2), reported the retention times of C_{18}-sphingosine, C_{18}-dihydrosphingosine, and 3-*O*-methyl-C_{18}-sphingosine on a 2.5% SE-30 (methyl silicone) column. Karlsson (3), using a different procedure for trimethylsilylation and a 6% silicone column, reported in graphical form the retention times of various homologues of sphingosine, dihydrosphingosine, and phytosphingosine as well as of their hydrolytic by-products. However, no quantitative application of the trimethylsilylation procedure has been made. In this paper we describe an improved reagent for the trimethylsilylation of LCB. The solution of TMSi derivatives can be analyzed quantitatively by gas–liquid chromatography up to several days after its preparation. We also report the retention times of all the common LCB, including the C_{20}-homologues and the anhydro bases, on a commercially available SE-30 column. Separation of the *erythro* and *threo* isomers of sphingosine and dihydrosphingosine as their *N*-acetyl TMSi derivatives is also described.

Abbreviations: ECL, equivalent chain lengths; LCB, long-chain base(s); TMSi, trimethylsilyl ether(s).

METHODS AND MATERIALS

An F & M (F & M Scientific Corp., Avondale, Pa.) Model 400 gas chromatograph with a hydrogen flame detector was used. The column packing consisted of 3.8% SE-30 on 80–100 mesh Diatoport S, obtained from F & M packed in a 180 cm × 0.4 cm I.D., U-shaped glass column. The column contained 3700 theoretical plates. The carrier gas was helium at 40 psi and flow rate of 60 ml/min. The flow rate of hydrogen (at 10 psi) was 30 ml/min, and that of compressed air (at 10 psi) was 280 ml/min. The flash heater and detector temperatures were kept 20–30°C higher than the column temperature. The column temperature was 210°C, 220°C, or 230°C, depending on the mixture of LCB. Areas were calculated from peak height times width at half height.

Synthetic C_{18}-sphingosine and C_{18}-dihydrosphingosine were obtained from Yeda Research and Development Co., Ltd., Rehovoth, Israel. Natural C_{18}-phytosphingosine and C_{18}-dehydrophytosphingosine were obtained from corn and flax, respectively, by procedures previously published by this laboratory (4). Synthetic C_{20}-phytosphingosine, as the *N*-benzoyl derivative, was the generous gift of Dr. Roy Gigg, National Institute for Medical Research, London, England. Synthetic C_{20}-sphingosine, as the oxalate salt, was obtained through the generosity of Dr. M. Prostenik, Chemical Institute of the Medical

School, Zagreb, Yugoslavia. *threo*-C_{18}-Sphingosine and *threo*-C_{18}-dihydrosphingosine were the generous gifts of Dr. E. F. Jenny, Ciba Ltd., Basel, Switzerland. 3-*O*-Methyl-C_{18}-sphingosine, 3-*O*-methyl-C_{18}-dihydrosphingosine, and sphingine (2-amino-octadecanol) were obtained from this laboratory. Anhydro-C_{18}-phytosphingosine and anhydro-C_{18}-dehydrophytosphingosine were isolated from hydrolysates of flax phytoglycolipid. The methods used for their isolation and purification and the evidence for their identification will be presented in a future paper. C_{20}-Dihydrosphingosine was obtained by catalytic hydrogenation of synthetic C_{20}-sphingosine with 5% palladium on charcoal. Free LCB were obtained from derivatives by hydrolysis with 1 N CH_3OH–HCl followed by extraction from an alkaline aqueous solution with diethyl ether. Stock solutions of the various bases were made in benzene–methanol 1:1.

The *N*-acetyl derivatives of the LCB were prepared by treating the free LCB (100–200 μg) with 50 μl of freshly prepared methanol–acetic anhydride 4:1 overnight at room temperature. Water (50 μl) was added, and after 1 hr the mixture was lyophilized.

Hexamethyldisilazane and trimethylchlorosilane were obtained from Applied Science Laboratories Inc., State College, Pa. Pyridine was distilled over barium oxide and stored over potassium hydroxide. Small vials (12 × 135 mm) with Teflon-lined screw caps were purchased from Arthur H. Thomas Co., Philadelphia, Pa. Tetracosane was obtained from Lachat Chemicals, Inc., Chicago, Ill.

The original silylation reagent was prepared according to Sweeley, Bentley, Makita, and Wells (2) by the addition of hexamethyldisilazane (2.0 ml) and trimethylchlorosilane (1.0 ml) to dry pyridine (10.0 ml). Removal of the white precipitate by centrifugation gave a clear, colorless supernatant solution which will be referred to as the "centrifuged original silylation reagent."

An "improved reagent for silylation" was prepared as follows. Hexamethyldisilazane (2.6 ml) was added to dry pyridine (2.0 ml) and mixed. Trimethylchlorosilane (1.6 ml) was then added and the mixture was shaken. The opaque solution was centrifuged to give a clear colorless supernatant solution which was stored in the dark. This solution (50 μl) was added to samples of dry solvent-free LCB (150 μg) in small vials with Teflon-lined caps. The reagent can be used for several weeks after preparation.

RESULTS

Use of the Original Silylation Reagent

In preliminary experiments on the distribution of LCB from various sources the original silylation reagent was added to mixtures of LCB and aliquots were injected at various times after the addition of the reagent. When aliquots were injected over a short time period following the addition of the reagent, the areas of the peaks were directly proportional to the quantity of material injected. However, the peak areas decreased as the time after the addition of the reagent increased. We then examined C_{18}-dihydrosphingosine mixed with tetracosane as an internal standard. The area and height ratios of the peaks were determined at various times from 5 min up to 5 hr after the addition of the original silylation reagent. The ratios were quite variable. On standing overnight, the originally cloudy white mixture gave a white precipitate and a clear supernatant solution. Injection of aliquots of the clear solution gave constant ratios. However, when the solution was remixed variable ratios were again observed. In all cases the area of the tetracosane peak per microliter injected remained essentially constant.

In view of the above results we decided to centrifuge the original silylation reagent and to use only the clear supernatant solution for preparation of the TMSi-LCB. In this experiment a mixture of C_{18}-sphingosine, C_{18}-dihydrosphingosine, and tetracosane was used. The height ratios of LCB/tetracosane were determined at various times from 5 min up to 24 hr after the addition of the silylation reagent. Height ratios were chosen so that errors in the measurement of small peak widths were eliminated. This centrifuged reagent gave reproducible and constant height ratios up to 150 min after the addition of the reagent and appeared to be usable for at least 6 days after its preparation.

The reproducibility of the method was checked with known mixtures of tetracosane (150 μg) and C_{18}-dihydrosphingosine (100, 150, and 200 μg). Portions of nine separate mixtures were injected 10 min after the addition of the reagent, and the area ratios of dihydrosphingosine/tetracosane were calculated. The average *K* value (area dihydrosphingosine/area tetracosane)/(wt dihydrosphingosine/wt tetracosane) was 0.92 ± 0.01.

At a range setting of 10 and attenuation of 4 with a column temperature of 210°C, 0.1 μg of C_{18}-dihydrosphingosine gave a peak which was 20% of full scale. Thus as little as 5 μg of LCB dissolved in 50 μl of reagent could be detected. This is about 0.015 μmole, close to the lower limit of the Lauter-Trams colorimetric procedure for LCB (5).

Use of the Improved Reagent for Trimethylsilylation

Although the centrifuged original silylation reagent gave reproducible results up to 150 min after its addition to the LCB, we thought it would be useful to have the derivatives remain stable for longer periods of time. This was achieved with the "improved reagent" for trimethyl-

TABLE 1 PEAK-HEIGHT RATIOS AND RELATIVE AREA PERCENTAGES OF A MIXTURE OF TRIMETHYLSILYL ETHER DERIVATIVES OF LONG-CHAIN BASES AS A FUNCTION OF TIME FOLLOWING TREATMENT WITH THE "IMPROVED TRIMETHYLSILYLATION REAGENT"

Time	Sphingine	S	SH_2	DP	P
			*Peak-Height Ratios**		
10 Min	1.10	1.48	1.47	1.02	1.03
70 Min	1.08	1.45	1.45	1.02	1.04
32 Hr	1.04	1.43	1.44	1.00	1.03
2 Days	1.08	1.44	1.46	1.03	1.06
6 Days	0.89	1.26	1.29	0.91	0.91
9 Days	0.88	1.28	1.30	0.87	0.88
			Relative Area Percentages		
10 Min	10.3	19.4	22.0	22.9	25.3
70 Min	10.2	19.2	21.9	23.0	25.6
32 Hrs	9.5	20.5	22.4	22.9	24.5
2 Days	9.8	19.9	22.4	22.5	25.3
6 Days	8.8	20.1	21.9	23.7	25.5
9 Days	8.9	20.4	23.4	22.6	24.8

A mixture of sphingine, C_{18}-sphingosine (S), C_{18}-dihydrosphingosine (SH_2), C_{18}-dehydrophytosphingosine (DP), C_{18}-phytosphingosine (P), and tetracosane was treated with the "improved silylation reagent." Aliquots of the reaction mixture were removed at various times after the addition of the reagent and subjected to gas–liquid chromatography on an SE-30 column.

* Peak height ratio = LCB/tetracosane.

TABLE 2 RELATIVE RETENTION TIMES AND EQUIVALENT CHAIN LENGTHS OF TRIMETHYLSILYL ETHER DERIVATIVES OF LONG-CHAIN BASES SEPARATED BY GAS–LIQUID CHROMATOGRAPHY ON AN SE-30 COLUMN

Compound	Relative Retention Time 210°C	220°C	230°C	Equivalent Chain Length
Sphingine	0.53	—	—	16.10
Anhydro-C_{18}-dehydrophytosphingosine	—	0.66	0.69	16.75
Anhydro-C_{18}-phytosphingosine	—	0.72	0.74	17.00
3-*O*-Methyl-C_{18}-sphingosine	0.73	—	—	17.10
3-*O*-Methyl-C_{18}-dihydrosphingosine	0.80	—	—	17.35
C_{18}-Sphingosine	0.88	0.88	0.89	17.65
C_{18}-Dihydrosphingosine*	1.00	1.00	1.00	18.00
C_{18}-Dehydrophytosphingosine	1.51	1.48	1.45	19.20
C_{18}-Phytosphingosine	1.65	1.61	1.56	19.45
C_{20}-Sphingosine	1.76	1.71	1.66	19.65
C_{20}-Dihydrosphingosine	2.00	—	1.87	20.00
C_{20}-Phytosphingosine	3.32	3.11	2.88	21.45
19-Methyl-C_{20}-phytosphingosine	—	—	3.58	22.10

* At 210 °C C_{18}-dihydrosphingosine had a retention time of 32 min while at 230 °C its retention time was 15 min.

silylation. Mixtures of sphingosine, dihydrosphingosine, phytosphingosine, dehydrophytosphingosine, and tetracosane were treated with the "improved reagent," and aliquots were injected at various time intervals from 10 min to 24 hr after the addition of the reagent. Reproducible and constant peak height ratios were obtained up to 24 hr. The same peak height ratios were obtained 5 min after the addition of the centrifuged original silylation reagent to these same LCB mixtures.

Finally a mixture of sphingine, C_{18}-sphingosine, C_{18}-dihydrosphingosine, C_{18}-phytosphingosine, C_{18}-dehydrophytosphingosine, and tetracosane was treated with the "improved reagent" and injected at various time intervals from 10 min to 9 days after the addition of the reagent. The solution was stored in a desiccated bottle in the refrigerator. The peak height ratios are shown in Table 1. The ratios of LCB/tetracosane were constant up to 2 days after the addition of the reagent. Although the peak height ratios decreased after 2 days, the relative area percentages of the various LCB remained essentially constant up to 9 days after the addition of the reagent (Table 1). Similar results were obtained in previous experiments with the "improved reagent" in which the peak height ratios of one LCB relative to another LCB remained constant while the ratio of LCB/tetracosane decreased with time.

Retention Times of the Various LCB

Data from the above experiments were also used to calculate the retention times of the various LCB relative to that of C_{18}-dihydrosphingosine. The averages and standard deviations obtained from eight determinations were the following: sphingine (0.527 ± 0.003), C_{18}-sphingosine (0.877 ± 0.003), C_{18}-dehydrophytosphingosine (1.505 ± 0.004), C_{18}-phytosphingosine (1.648 ± 0.005). The retention times of other LCB, expressed in terms of equivalent chain lengths (ECL) relative to C_{18}- and C_{20}-dihydrosphingosine are given in Table 2. At a column temperature of 210°C, C_{20}-sphingosine and C_{20}-phytosphingosine had exactly twice the retention times of the C_{18}-homologues; at 230°C the retention times of the C_{20}-LCB are 1.86 times those of the C_{18}-LCB. From these values, log retention time vs. carbon number plots can be made and used for the calculation of the retention times of other homologues. For example, at 210°C C_{17}- and C_{19}-dihydrosphingosine would have calculated retention times of 0.70 and 1.40 respectively, relative to C_{18}-dihydrosphingosine.

Separation of erythro and threo Isomers

The TMSi derivatives of the *erythro* and *threo* isomers of a particular LCB are eluted together from the SE-30 column. However, the *erythro* and *threo* isomers can be separated on the SE-30 column if the *N*-acetyl TMSi derivative is used. This separation was reported recently (6), but no data were given regarding the retention times of the various isomers. The retention times of the various *N*-acetyl TMSi isomers of sphingosine and dihydrosphingosine are given in Table 3. The TMSi derivative

TABLE 3 RELATIVE RETENTION TIMES OF *N*-ACETYL TRIMETHYLSILYL ETHER DERIVATIVES OF VARIOUS LONG-CHAIN BASES SEPARATED BY GAS–LIQUID CHROMATOGRAPHY ON AN SE-30 COLUMN

N-Acetyl TMSi Compound	Retention Time at 220°C	
	Relative to SH_2*	Relative to *erythro*-*N*-$AcSH_2$†
threo-C_{18}-Sphingosine	1.71	0.82
threo-C_{18}-Dihydrosphingosine	1.80	0.86
erythro-C_{18}-Sphingosine	1.88	0.90
erythro-C_{18}-Dihydrosphingosine	2.09	1.00
threo-C_{20}-Sphingosine	3.26	1.56
erythro-C_{20}-Sphingosine	3.58	1.72

* SH_2, C_{18}-dihydrosphingosine.
† *N*-$AcSH_2$, *N*-acetyl C_{18}-dihydrosphingosine.

of synthetic C_{20}-sphingosine gave one peak, whereas the *N*-acetyl TMSi derivative gave two peaks about equal in area. On thin-layer chromatography with $CHCl_3$-CH_3OH–NH_4OH 100:25:2.5 and Silica Gel G-HR, the free LCB gave two closely separated spots of about equal intensity. The C_{18}-phytosphingosine and C_{18}-dehydrophytosphingosine samples used in this study gave one peak each, both as the TMSi and as the *N*-acetyl TMSi derivatives. No evidence was found for the presence of unacetylated LCB after treatment with methanol–acetic anhydride 4:1.

DISCUSSION

The modified reagent for trimethylsilylation described in this paper represents an improvement over the original silylation reagent. The "improved reagent" gives TMSi derivatives, stable for at least 2 days, of all of the common LCB and makes it possible to determine the composition of a mixture of bases up to 1 wk after the preparation of the derivatives. In the original silylation reagent the approximate molar proportions of pyridine–hexamethyldisilazane–trimethylchlorosilane were 940:73:61. In the "improved reagent" the proportions are 390:200:200. Although it is not clear why the reagent is superior, the increased proportions of hexamethyldisilazane and trimethylchlorosilane may protect the TMSi-LCB by reacting with traces of water which might otherwise cause hydrolysis of the ethers.

Several experiments were performed in which only hexamethyldisilazane and pyridine were used with mixtures of LCB, in an attempt to form the TMSi-LCB without the formation of HCl. At room temperature only small quantities of the TMSi-LCB were formed after 1.5 hr. Heating the mixture for 15 hr at 65–70°C did give significant quantities of the derivatives, as indicated by gas chromatography, but an extra peak was found between sphingosine and dihydrosphingosine. Further work along these lines was not continued, since the rapidity of the reaction at room temperature made the mixture of hexamethyldisilazane and trimethylchlorosilane the choice reagent.

The relative retention times of all of the common LCB, given in Table 2, are easily reproducible to within ±0.01 even when determined several months apart, provided of course that the same liquid phase, column temperature, and other gas chromatographic conditions are used. However, relative retention times have the disadvantage of being temperature-dependent as is illustrated in Table 2. The equivalent chain lengths (ECL) on the other hand are the same whether determined at 210 or 240°C. C_{18}-Dihydrosphingosine was chosen as the reference LCB simply because it is available commercially.

Although gas–liquid chromatography of the TMSi-LCB provides a rapid and sensitive method for the determination of various LCB, retention time data or ECL data alone simply indicate what a particular LCB may or may not be: they do not positively identify the LCB. However, when considered along with other data, such as the aldehydes liberated following oxidation with periodate (7), this method provides strong supporting evidence for the structure of a particular LCB. As an example, C_{18}-dihydrosphingosine was reported to be present in cerebrin from *Torulopsis utilis* (8), the conclusion being based on the identification of hexadecanal following periodate oxidation. However, this aldehyde could also have resulted from the periodate oxidation of C_{19}-phytosphingosine. Gas chromatography of the TMSi-LCB provided a rapid means of determining which of these LCB was indeed present. We confirmed the finding (9) that treatment of LCB from yeast cerebrin (Nutritional Biochemicals Corporation, Cleveland, Ohio) with periodate did yield hexadecanal. However, gas–liquid chromatography of the TMSi-LCB demonstrated the presence of C_{19}-phytosphingosine and not C_{18}-dihydrosphingosine. The presence of C_{19}-phytosphingosine in yeast cerebrin along with the C_{18}- and C_{20}-homologues makes this material a good source of a homologous series of LCB which can be used to obtain data for the construction of a log retention time vs. carbon number plot.

Although most of the common LCB can be separated by gas chromatography of the TMSi-LCB, the anhydrophytosphingosines do have the same retention times as the dihydrosphingosines containing one less carbon atom (see Table 2). These compounds are, however, readily separated on silicic acid.

Gas chromatography of the TMSi derivatives of the *N*-acetyl LCB provides a simple and rapid method for the separation and quantification of the *erythro* and *threo* isomers of the sphingosine series. Although good separations are obtained with the individual LCB, some of the isomers are not well separated when mixtures of

various *N*-acetyl-LCB are used. Compare, for example, the separation of *threo*-*N*-acetyl-sphingosine and *threo*-*N*-acetyl-dihydrosphingosine shown in Table 3. Nevertheless, this procedure should be useful in determining the proportions of *erythro* and *threo* isomers present in the product of a chemical synthesis of a LCB, in commercial preparations, and in purified LCB from natural sources. It would also be a useful tool in a study of conditions which lead to inversion of configuration and in determining the stereochemistry of a new LCB.

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