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PREPARATION OF SPHINGOLIPID FATTY ACID METHYL ESTERS FOR DETERMINATION BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

Sphingolipid fatty acids are first converted to a mixture of free acids and their *n*-butyl esters by heating the specimen at 85°C in aqueous butanolic hydrogen chloride; the butyl esters are then saponified with methanolic potassium hydroxide. After acidification and extraction into hexane, the fatty acids are extracted into a very small volume of aqueous trimethyl(*m*-trifluorotolyl)ammonium hydroxide (TMTFTH), injection of an aliquot of the TMTFTH extract into the gas chromatograph yields the fatty acid methyl esters by pyrolytic methylation of the quaternary ammonium salts of the fatty acids. The preparation of a specimen ready for gas–liquid chromatographic (GLC) analysis with quantitative recovery of the sphingolipid fatty acids can be accomplished in less than 2 h. By comparison, none of a number of well-accepted techniques for the release of sphingomyelin fatty acids by hydrolysis or methanolysis released the fatty acids quantitatively in less than 3 h, and all required additional manipulations before GLC analysis.

INTRODUCTION

Preparation of fatty acid methyl esters from the total saponifiable fatty acids of biological specimens or lipid fractions is readily achieved by a number of means, which can include direct methylation of isolated lipids by alkaline or acidic methanol or methylation of the fatty acids isolated after saponification. The saponifiable fatty acids include free fatty acids and fatty acids from esters of glycerol, sterols and a variety of other alcohols. Sphingolipids, however, are extremely resistant to alkaline treatment because the sphingolipid fatty acids exist as amides rather than esters, and only undergo acid hydrolysis or acid methanolysis very slowly.

A new procedure for preparing fatty acid methyl esters from the fatty acid amides of sphingolipids has been developed. The specimen can be prepared ready for gas–liquid chromatographic (GLC) analysis from an isolated lipid fraction in less than 2 h, with quantitative conversion of the sphingolipid fatty acids to their methyl

esters. The new procedure has been compared with a number of commonly used techniques, none of which released all of the fatty acids in 2 h and all of which required additional manipulations before the specimen could be subjected to GLC analysis. Reaction of sphingomyelin for 2 h in each of the following reagents did not yield quantitative release of the fatty acids: 2 *N* aqueous hydrogen chloride at 110°C (ref. 1) released 86% of the fatty acids; 3 *N* methanolic hydrogen chloride at 100°C (ref. 2) released 84%; methanol–sulphuric acid–benzene (100:5:5) at 70°C (ref. 3) released 60%; and 14% boron trifluoride solution in methanol at 100°C (ref. 4) released 25%.

In addition to the saving in time, the proposed procedure has additional advantages over other procedures. Anhydrous conditions are not necessary. The fatty acids only, free of neutral or basic lipid-digestion products, are subjected to GLC analysis. Flame-sealed ampoules, PTFE-lined screw-capped tubes, or other special glassware are not necessary — all the manipulations are performed in two centrifuge tubes, with only one solvent transfer. No evaporation steps are necessary. All the reagents are readily available. Prolonged heating in acidic reagents at high temperatures is not necessary.

EXPERIMENTAL

Reagents

Sphingolipids and free fatty acids were obtained from Sigma (St. Louis, MO 63178, U.S.A.) and Applied Science Labs. (State College, PA, U.S.A.). All reagents and solvents used were the best grades available.

Wet butanolic hydrogen chloride. To two volumes of *n*-butyl alcohol were added one volume of water and one volume of concentrated hydrochloric acid (36.5–38%), with mixing after each addition; a one-phase reagent was obtained, which was stored at room temperature in a stoppered container.

TMTFTH, 0.5 M. Aqueous 0.5 *M* trimethyl(*m*-trifluorotolyl)ammonium hydroxide (TMTFTH) was prepared as described by MacGee and Allen[5]. Applied Science Labs. supplies aqueous 0.2 *M* TMTFTH as Meth-Prep I; Regis (Morton Grove, IL, U.S.A.) supplies aqueous 0.5 *M* TMTFTH. Note that Meth-Prep I is aqueous 0.2 *M* TMTFTH, and thus the total amount of fatty acid that can be extracted from the hexane will be *ca.* two-fifths of the amount that can be extracted with 0.5 *M* TMTFTH.

Methyl acetate, sodium carbonate-treated. Methyl acetate (5 ml) and 1 g of anhydrous sodium carbonate were shaken well in a polyethylene-stoppered test-tube, and the salt was allowed to settle; the mixture was stored at room temperature in a stoppered container.

Methanolic potassium hydroxide, 10%. Potassium hydroxide (50 g) was dissolved in *ca.* 300 ml of methanol and diluted with methanol to 500 ml at room temperature. After standing overnight, the clear solution was decanted from any potassium carbonate in the flask. This reagent was stored at room temperature in a PTFE-stoppered glass reagent bottle.

Phosphoric acid, 1 M. Phosphoric acid (85%; 34 ml) was added to *ca.* 300 ml of distilled water and diluted to 500 ml with distilled water at room temperature.

Chromatography

A Bendix 2600 gas chromatograph (Bendix, Ronceverte, WV 24970, U.S.A.) with flame ionization detectors and temperature programming was used with a 6 ft. × 1/4 in. (4 mm I.D.) glass column packed with pretested 10% Silar 10C on 100–120 mesh Gas-Chrom Q (Applied Science Labs.). The injector and detector were kept at 250°C, and nitrogen at 32 ml/min was the carrier gas. The column oven was temperature programmed from 160°C to 220°C at 2°C/min. The peak areas and composition were determined with an Autolab System I computing integrator (Spectra-Physics, Santa Clara, CA, U.S.A.).

A Perkin-Elmer Sigma I microprocessor-controlled computing gas-chromatographic system was used, with flame detectors and a 2 m × 1/8 in. (2 mm I.D.) nickel column packed with 10% Silar 10C on 100–120 mesh Gas-Chrom P (Applied Science Labs.). The injector and detector were kept at 250°C, and the oven was temperature programmed at 2°C/min from 160°C to 220°C. Nitrogen at 10ml/min was the carrier gas.

Procedures

Isolated lipid fractions. An aliquot of the lipid containing not more than 100 μg of total fatty acids was mixed with a known amount of margaric acid (Applied Science Labs.) (between 10 and 40 μg) in chloroform–methanol (5:1, v/v) and evaporated to dryness with a stream of nitrogen in a 15-ml glass-stoppered conical centrifuge tube. Wet butanolic hydrogen chloride (0.1 ml) was added, and, after stoppering and mixing well on a vortex mixer (Scientific Products, Obetz, OH, U.S.A.), the mixture was heated at 85°C for 80 min in a Temp Blok Heater (Scientific Products). After cooling, 0.5 ml of 10% methanolic potassium hydroxide was added, and the contents of the tube were mixed well on the vortex mixer and were then heated for 30 min at 65°C in a Temp Blok Heater; then 0.5 ml of 1 *M* phosphoric acid were added, and the tube was lightly shaken. With the addition of 5 ml of hexane, the tube was stoppered and shaken vigorously by hand for 1 min. Before centrifugation, 5 ml of distilled water were added to the tube, and the tube was gently tilted for 1 min. After centrifugation at 600 *g* for 1 min, nearly all of the clear upper hexane phase was transferred with a pasteur pipette to a 5-ml glass-stoppered conical centrifuge tube. It was not necessary to transfer all of the extract, but care was taken to avoid transfer of any of the lower phase or the emulsion layer. To the extract were added 10 μl of 0.5 *M* TMTFTH, and the stoppered tube was shaken vigorously by hand for 1 min, care being taken that none of either phase was trapped in the tip of the tube during the shaking (otherwise extraction would be incomplete). After centrifugation at 600 *g* for 1 min, a 1–3 μl aliquot of the lower TMTFTH layer was removed with a 10-μl microsyringe pre-wetted with sodium carbonate-treated methyl acetate. A 1-μl aliquot of the treated methyl acetate was drawn into the syringe after the TMTFTH extract, and the syringe plunger was pumped back and forth several times before injection of the liquid into the GLC unit. For optimal results, it was necessary to determine whether rapid or slow injection was better for each GLC unit. The widths of the solvent–reagent peaks and the heights of the fatty acid methyl ester peaks can vary considerably with injection rate, carrier-gas flow-rate and the geometry of the inlet system of the GLC unit. The injection rate should be varied from very rapid to very slow (up to 5 sec per μl) to determine the optimal rate for a given system.

Time studies. A chloroform–methanol (5:1, v/v) solution containing, per ml, 2 mg of sphingomyelin and 0.6 mg of margaric acid was used in the following study to determine the time courses of the heating step in our method and in the conventional methods. Chromatography was performed on the Perkin-Elmer Sigma I unit.

Aliquots (each 25 μl) of the sphingomyelin–margaric acid solution were evaporated and subjected to incubation with wet butanolic hydrogen chloride at 85°C for various periods; they were then carried through the rest of the procedure as described above. For comparison, aliquots of the same sphingomyelin–margaric acid solution were treated by four conventional methods[1–4].

Following the method proposed by Morrison and Smith[4], 1-ml aliquots of the sphingomyelin–margaric acid were evaporated in 15-ml PTFE-lined screw-capped conical centrifuge tubes, 0.5 ml of 14% boron trifluoride in methanol being added, under nitrogen, before tightly securing the cap. The tubes were then incubated at 100°C for various periods, and, after heating, were cooled under running tap water. The reaction mixture was then shaken vigorously with 1 ml of aqueous saturated sodium chloride and 5 ml of hexane. After allowing the phases to separate, the hexane extract was removed from the tube and dried with anhydrous sodium sulphate. The sample was evaporated with a stream of nitrogen, and the products were reconstituted for GLC in 1,1,2-trichloroethane.

For heating with methanolic hydrogen chloride, 3 *N* (Supelco, Bellefonte, PA, U.S.A.) as suggested by Sweeley and Moscatelli[2], 2.0-ml aliquots of the sphingomyelin–margaric acid solution were evaporated in 15-ml PTFE-lined screw-capped conical centrifuge tubes. The tubes were sealed tightly after adding 0.5 ml of the methanolic hydrogen chloride reagent and were incubated at 100°C for the times indicated in Fig. 1. The fatty acid methyl esters were reconstituted in 1,1,2-trichloroethane by the procedure described above.

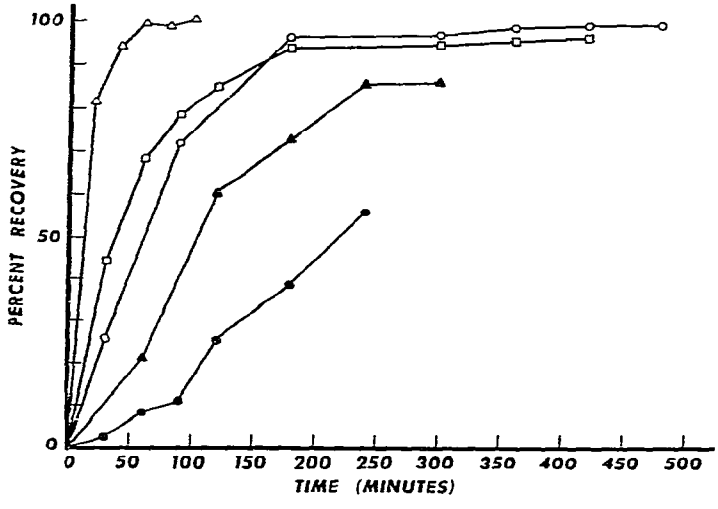

Fig. 1. Time course of release of sphingomyelin fatty acids by various procedures. △, butanol–HCl–H_2O (2:1:1), heated at 85°C; ○, 3 *N* methanolic HCl heated at 100°C; □, 2 *N* aqueous HCl heated at 110°C; ▲, methanol–H_2SO_4–benzene (100:5:5) heated at 70°C; ●, 14% BF_3 in methanol heated at 100°C.

Aliquots (1 ml) of the sphingomyelin–margaric acid solution were evaporated in 15-ml PTFE-lined screw-capped tubes, and the residues were incubated with 0.5 ml of methanol–sulphuric acid–benzene (100:5:5, v/v/v) at 70°C for various periods of time[3]. The fatty acid methyl esters were reconstituted in 1,1,2-trichloroethane by the procedure described above.

For the procedure described by O'Brien *et al.*[1], 1.0-ml aliquots of the sphingomyelin–margaric acid solution were evaporated in ampoules. The ampoules were sealed after the addition of 1.0 ml of 2 *N* aqueous hydrochloric acid and incubated, with frequent vortexing, at 110°C for various periods of time. The ampoules were cooled under running tap water, and the contents were transferred quantitatively to a culture tube (100 × 12 mm). To isolate the now free fatty acids and dispense with any further heating, we proceeded with the hexane and quaternary ammonium extraction of our new procedure as described above. Hexane (5 ml) was added, and the tube was capped with a polyethylene cap and shaken vigorously. After centrifugation at 600 *g* for 1 min, the hexane phase was transferred to a 5.0-ml glass-stoppered conical centrifuge tube, and the free fatty acids were extracted into 10.0 μl of 0.5 *M* TMTFTH as described above in the new procedure.

Precision studies. Aliquots of 100 μg of sphingomyelin in chloroform–methanol (5:1, v/v) were subjected to the isolated lipid procedure, and the methyl esters were chromatographed on the Silar 10C column in the Bendix unit. The means and standard deviations of the normalized percentage composition (the percentage of the total peak area) were compared with reactions in methanolic hydrogen chloride at 100°C for 6 h (ref. 2), followed by saponification, acidification, hexane extraction and final extraction into TMTFTH[5] and with a set of methanolic hydrogen chloride reactions in which the methyl esters were obtained in 1,1,2-trichloroethane as described above.

RESULTS AND DISCUSSION

Fig. 1 shows the release of stearic acid as measured relative to margaric acid (the internal standard) with time when the sphingomyelin–margaric acid solution was treated by the various heating steps (*Time studies*); stearic acid was the major fatty acid in this specimen of sphingomyelin. Fig. 1 shows that release of stearic acid was complete in much less than 80 min at 85°C by the new proposed procedure, whereas the other conventional procedures took considerably longer. It appears from Fig. 1 that the heating times recommended in the literature for some of these conventional procedures are insufficient.

The results of the precision studies are shown in Table I. For comparison, three procedures were used; our new procedure, the methanolic hydrogen chloride procedure with conventional preparation of fatty acid methyl esters, and the same procedure with preparation of saponified fatty acids only in TMTFTH[5]. Since the chromatograms of fatty acid methyl esters prepared by the conventional procedures contain peaks of non-fatty acid substances, a comparison with chromatograms containing only fatty acid peaks was necessary to ensure comparison of the same substances. The presence of non-fatty acid substances was minimal in these specimens, so the differences between the two sets of methanolic hydrogen chloride-treated samples are small.

As can be seen from the comparison of these three procedures, there is ex-

TABLE I

COMPARISON OF THREE PROCEDURES FOR RECOVERY OF SPHINGOMYELIN FATTY ACIDS

Preparation of fatty acids for GLC for the column labelled "*3 N Methanol–HCl A*" was by saponification and final isolation of fatty acids in TMTFTH, whereas, for the column labelled "*3 N Methanol–HCl B*", the fatty acid methyl esters were isolated in trichloroethane (see text for more details). The data are expressed as percentages of the total peak area ($n = 8$).

Acid	*3 N Methanol–HCl A*	*n-Butanol–HCl–H_2O*	*3 N Methanol–HCl B*
16:0	3.30 ± 1.121	2.88 ± 0.152	2.15 ± 0.465
18:0	38.72 ± 3.420	40.27 ± 3.750	37.86 ± 5.378
20:0	0.59 ± 0.055	0.59 ± 0.046	0.58 ± 0.078
22:0	2.60 ± 0.108	2.52 ± 0.078	2.61 ± 0.119
23:0	2.53 ± 0.174	2.41 ± 0.107	2.59 ± 0.115
24:0	9.21 ± 1.490	9.04 ± 0.56	9.76 ± 1.058
24:1	32.30 ± 2.484	30.24 ± 1.467	32.17 ± 2.636
Unknown 1	2.94 ± 0.584	2.84 ± 0.178	3.09 ± 0.563
Unknown 2	4.19 ± 0.490	3.87 ± 0.271	4.28 ± 0.656
Unknown 3	0.88 ± 0.246	0.94 ± 0.092	1.02 ± 0.240
Unknown 4	4.11 ± 0.461	3.74 ± 0.442	4.08 ± 0.814

cellent agreement between the wet butanolic hydrogen chloride and the two methanolic hydrogen chloride sets. Also shown in Table I is the precision one can expect in this new procedure. The fatty acids have relative standard deviations of 3.0 to 11.8%. Thus, the proposed procedure is as precise as the accepted procedures.

Another advantage of the new procedure is the isolation of the sphingolipid fatty acids as free acids in a quaternary ammonium reagent. This advantage is twofold. First, only the acids are extracted from the organic phase into the very small volume of alkaline methylating agent; when this mixture is chromatographed, only fatty acids appear in the chromatogram (the elimination of interfering non-fatty acid peaks aids in accurate analysis). Secondly, the use of a very small volume of TMTFTH yields a solution sufficiently concentrated for direct injection into the gas chromatograph, *i.e.*, evaporation, a potentially destructive step, is not needed.

Differential analysis of sphingolipid fatty acids can be performed by determining the saponifiable fatty acids in one aliquot of a specimen and subjecting another aliquot to the procedure described here. Since our new procedure measures total fatty acids and our saponifiable fatty acid procedure[5] does not release sphingomyelin fatty acids (as can be seen from the zero time point in Fig. 1), addition of an internal standard allows the measurement of saponifiable fatty acids in one sample and total fatty acids in the other in terms that can be expressed as the weight of each fatty acid per weight of tissue, and sphingolipid fatty acids can be determined in the same units by difference. As an illustration, pentadecanoic acid was added at 10 μg/mg of wet tissue to a Folch extract[6] of cat spinal cord, and aliquots were subjected to the two procedures, but without additional pentadecanoic acid. A comparison of the chromatograms obtained for total fatty acids and saponifiable fatty acids is shown in Fig. 2. The results, expressed in μg of each fatty acid per mg of wet cord for total fatty acids, saponifiable acids and sphingolipid fatty acids (by difference) are shown in Table II. The precision in such an analysis will be slightly poorer than can be obtained from the normalized percentage composition because the measurement of

TABLE II

ANALYSIS OF FATTY ACIDS IN A FOLCH EXTRACT OF CAT SPINAL CORD

Values are expressed as μg/mg of wet cord. Total fatty acids and saponifiable fatty acids are experimentally obtained; sphingolipid fatty acids are derived by subtraction of saponifiable fatty acid from total fatty acid values.

Acid	*Total fatty acids*	*Saponifiable fatty acids*	*Sphingolipid fatty acids*
16:0	3.20	2.96	0.24
16:1	0.14	0.18	—
18:0	5.43	3.28	2.15
18:	8.08	8.54	—
18:2	0.10	0.12	—
20:0	0.64	0.07	0.57
18:3	2.66	2.72	—
22:0	0.64	0.19	0.45
22:1	0.73	0.64	0.09
24:0	2.37	0.00	2.37
24:1	4.21	0.00	4.21
X	0.58	0.36	0.22
22:5	0.08	0.03	0.05
22:6	0.72	0.26	0.46
Y	0.50	0.00	0.50

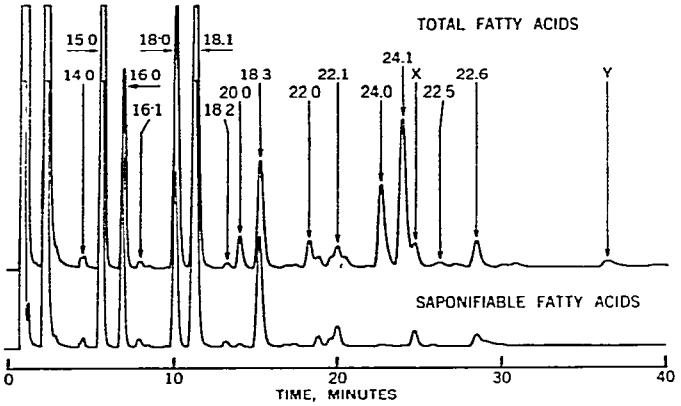

Fig. 2. Comparison of chromatograms of saponifiable fatty acids and total fatty acids of cat spinal cord.

each fatty acid involves any errors in measuring two peaks (the peak of interest and the internal-standard peak) and then obtaining the difference by subtraction. It should be noted that, unless there is a large amount of sphingolipid in the specimen relative to the total amount of lipid, the differences, although significant, will not be striking.

CONCLUSIONS

A rapid and simple method for the conversion of sphingolipid fatty acids to their methyl esters has been described. The results obtained with this new procedure

are comparable to the results obtained with properly used conventional procedures. Besides a considerable saving in time, this procedure offers other advantages. Also, when used in conjunction with our saponifiable fatty acid procedure[5], this procedure can yield important information on the amount of each saponifiable fatty acid and sphingolipid fatty acid per weight of tissue. We feel that, with this procedure, much useful information of biomedical importance can be obtained, especially since the procedure is rapid and requires only small samples.

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