Journal of Chromatography, 607 (1992) 55–67
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 329

Studies on the methanolysis of small amounts of purified phospholipids for gas chromatographic analysis of fatty acid methyl esters

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(First received January 7th, 1992; revised manuscript received April 22nd, 1992)

ABSTRACT

The methanolysis of small amounts of purified phosphoglycerides and sphingomyelin was studied and a quantitative comparison of five methods for the methanolysis of standard phosphoglycerides was made. These methods were based on methanolysis with boron trifluoride–methanol, methanolic sodium methoxide (at ambient temperature and with heating) and methanolic sulphuric acid. A further method was based on saponification with methanolic sodium hydroxide and subsequent esterification with boron trifluoride–methanol. Under the experimental conditions, only the sodium methoxide-catalysed method at ambient temperature gave complete methanolysis of phosphoglycerides. For methanolysis of sphingomyelin, boron trifluoride–methanol, methanolic sulphuric acid and methanolic hydrochloric acid were used. It was found that complete methanolysis of sphingomyelin takes 15 h at 90°C. Based on these results, procedures for the methanolysis of phosphoglycerides and sphingomyelin separated by high-performance liquid chromatography are presented.

INTRODUCTION

For the quantification of phospholipid classes in biological materials, thin-layer chromatographic separation with subsequent densitometric or spectrophotometric measurement of phospholipid phosphorus is the most widely used procedure [1–4]. Newer methods often measure phospholipids, which have been separated by thin-layer chromatography or high-performance liquid chromatography (HPLC), by determining total phospholipid-bound fatty acids by gas chromatography (GC) [5,6]. This procedure allows a more precise measurement of phospholipid classes than phosphorimetry, because the molecular mass of bound fatty acids is taken into consideration.

For the quantification of phospholipid classes by fatty acid analysis, a complete conversion of phospholipid-bound fatty acids into fatty acid methyl esters (FAMEs) is necessary. For methanolysis of phospholipids, many methods have been published. Most of them use boron trifluoride–methanol [7–10], diazomethane [11,12], methanolic sulphuric or hydrochloric acid [13,14], methanolic sodium methoxide [15–18] and quaternary ammonium hydroxides [19–21]. Other reagents for methanolysis, seldom used, include methanolic acetyl chloride [22], methanolic aluminium chloride [23] and methanolic methyl iodide [24]. There are also methods involving saponification with subsequent esterification [25,26]. All these methods for preparing FAMEs have been reviewed [27,28]. Moreover, studies comparing different methods have been published [12,29,30]. However, most of these methods have been applied to standards or samples containing milligrams to grams of triacylglycerols [27], and there is little information about the methanolysis of very small amounts of phospholipids. Therefore, in

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0021-9673/92/$05.00

this work various methods were applied to the methanolysis of phospholipid standards in small amounts in order to check the rate of conversion of bound fatty acids into FAMEs. In the first part of the study, various methods were used for the methanolysis of standard phosphoglycerides. In the second part, methanolysis of sphingomyelin was investigated by using various reagents and experimental conditions. This paper also presents methods for the methanolysis of phosphoglycerides and sphingomyelin from erythrocyte membranes in microgram amounts separated by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Materials

All chemicals were purchased from Merck (Darmstadt, Germany) with the exception of boron trifluoride–methanol reagent (100 g/l, puriss.), which was from Fluka (Buchs, Switzerland), and fatty acid and phospholipid standards, which were from Sigma (Taufkirchen, Germany). Phospholipid standards were phosphatidylcholine (PC, from egg yolk, hydrogenated), phosphatidylethanolamine (PE, synthetic, dipalmitoyl; and PE, from bovine brain, containing *ca.* 60% plasmalogen); phosphatidylserine (PS, from bovine brain), lyso-phosphatidylcholine (LPC, from egg yolk), lyso-phosphatidylethanolmine (LPE, from egg yolk) and sphingomyelin (SM, from bovine brain). A "phospholipid standard from egg" purchased from Merck was also used. Blood samples were taken from adult female Sprague–Dawley rats.

FAMEs were analysed using a GC system which consists of a Siemens (Karlsruhe, Germany) Sichromat 2 gas chromatograph equipped with a temperature-programmed vaporizing (PTV) injection system, a Chrompack (Middelburg, Netherlands) wall-coated open-tubular fused-silica (CP-Sil 88) column (50 m × 0.25 mm I.D., film thickness 0.2 μm), a flame ionization detector (FID) and a Merck–Hitachi D-2500 integrator. HPLC separation of erythrocyte membrane phospholipids was carried out with a Merck–Hitachi HPLC system (L-6200 intelligent pump, L-3000 multi-channel photodetector, D-2000 integrator) with a 25 cm × 0.4 cm I.D. Si 60 (5 μm) cartridge (LiChroCART, Merck) and a Model 201 fraction collector (Gilson, Villiers-le-Bel, France).

Extraction and separation of erythrocyte membrane phospholipids

Extraction and separation of erythrocyte membrane phospholipids were carried out as described by Eder *et al.* [6,31]. Erythrocytes were washed three times with physiological saline, lysed by addition of distilled water and freezing. Erythrocyte membranes were washed three times according to Hanahan and Ekholm [32]. Phospholipids were extracted by the method of Peuchant *et al.* [33] using isopropanol as solvent. Erythrocyte membrane phospholipid classes were separated by HPLC using a gradient system based on the mobile phases (a) acetonitrile, (b) acetonitrile–H_3PO_4 (99.8:0.02) and (c) methanol–H_3PO_4 (99.8:0.02). Using this separation method, the major phospholipid classes of erythrocyte membranes (PS, PE, PC and SM) could be separated. Moreover, plasmalogens could be separated from diacyl phosphoglycerides, as plasmalogens were hydrolysed by the acidic mobile phase and hence eluted as 2-acyl lyso analogues [34–36]. Therefore, all the phosphoglycerides mentioned were purified phospholipids, which exist either as diacylphosphoglycerides or as plasmalogens. A 100-μl aliquot of the lipid extract containing phospholipids from *ca.* 170 μl of packed erythrocytes was injected manually. Phospholipid classes which were detected at 205 nm were collected automatically with a fraction collector. The mobile phase was evaporated and phospholipids were dissolved in chloroform–methanol (1:1) for methanolysis.

Methanolysis procedures

Methanolysis of phosphoglyceride standards

Standard solutions of each of the phospholipids PC, PE, PS, LPC and LPE were prepared in chloroform–methanol (1:1), containing butylated hydroxytoluene (BHT) at similar concentrations (0.5–1 mg/ml). A solution of "phospholipids from egg" was prepared in the same solvent at a concentration of 4.6 mg/ml. For investigation of methanolysis of each of these preparations, test tubes with PTFE-lined caps were used. Prior to methanolysis, a

known amount of methyl heptadecanoate (dissolved in *n*-hexane), as internal standard, was pipetted into the test-tube and the solvent removed by vacuum. After addition of the sample to be methanolysed, and before closing, the tubes were flushed with nitrogen. Methanolysis was carried out by one of the procedures A–E detailed below. On completion of the methanolysis reaction, 0.5 ml of water was added to the reaction mixture and the FAMEs were extracted twice with 0.5-ml amounts of *n*-hexane. After separation from the aqueous phase, the pooled hexane layers contained the FAMEs, which were then suitable for GC analysis.

Procedure A was a modification of the method of Morrison and Smith [7]: 1 ml of phospholipid solution and 2 ml of boron trifluoride–methanol reagent were added to the tubes containing methyl heptadecanoate. The solution was heated at 90°C for 30 min, cooled and the FAMEs were extracted.

Procedure B was a modification of the method of Butte [20]: 1 ml of phospholipid solution and 1 ml of 1 *M* methanolic sodium methoxide solution were added to the tubes containing methyl heptadecanoate. The tubes were heated at 75°C for 20 min, cooled and the FAMEs were extracted.

Procedure C was a modification of the method of Olegard and Svennerholm [37]: 1 ml of phospholipid solution and 1 ml of 1 *M* methanolic sodium methoxide solution were added to the tubes containing methyl heptadecanoate and the tubes were shaken at ambient temperature for 1 h, then the FAMEs were extracted.

Procedure D: 1 ml of phospholipid solution and 2 ml of 6% methanolic sulphuric acid were added to the tubes containing methyl heptadecanoate. The tubes were heated at 90°C for 2 h, cooled and the FAMEs were extracted.

Procedure E was a modification of the method of Slover and Lanza [25]: 1 ml of phospholipid solution and 1 ml of 1 *M* methanolic sodium hydroxide solution were added to the tubes containing methyl heptadecanoate. The tubes were heated at 90°C for 15 min. After the tubes had cooled, 2 ml of boron trifluoride–methanol reagent were added and the tubes were heated at 90°C for 15 min. The tubes were cooled and the FAMEs were extracted.

Methanolysis of plasmalogens

A standard solution of "PE from bovine brain", which contained *ca.* 60% plasmalogens, was prepared in chloroform–methanol (1:1), containing BHT, at a concentration of *ca.* 1 mg/ml. For methanolysis of this standard, boron trifluoride–methanol (according to procedure A above), methanolic sodium methoxide (according to procedure C) and methanolic sulphuric acid (according to procedure D) were used.

Methanolysis of sphingomyelin standard

A standard was prepared by dissolving sphingomyelin in chloroform–methanol (1:1), containing BHT, at a concentration of *ca.* 1 mg/ml. For investigation of methanolysis, tubes with PTFE lined caps were used. After addition of the sample to be methanolysed, and before closing, the tubes were flushed with nitrogen. On completion of the methanolysis reaction, 0.3 ml of water was added to the reaction mixture and FAMEs were extracted twice with 0.3-ml amounts of *n*-hexane. After separation from the aqueous phase, the pooled hexane layers contained the FAMEs, which were then suitable for GC analysis.

Time and concentration studies

Boron trifluoride–methanol methanolysis. Into each test-tube, 0.5 ml of sphingomyelin solution, 0.2 ml of *n*-hexane containing methyl heptadecanoate and 1 ml of boron trifluoride–methanol reagent were pipetted. The tubes were heated at 90°C for 1, 2, 6 or 15 h.

Sulphuric acid–methanol methanolysis. Into each test-tube, 0.5 ml of sphingomyelin solution, 0.2 ml of *n*-hexane containing methyl heptadecanoate and 1 ml of 6% or 15% methanolic sulphuric acid were pipetted (giving sulphuric acid concentrations of 1 and 2.5 *M*). To obtain a one-phase system, 0.5 ml of chloroform was added. Into other test-tubes, 0.2 ml of *n*-hexane containing methyl heptadecanoate was pipetted and the solvent was evaporated. A 0.5-ml volume of sphingomyelin solution, 0.5 ml of chloroform and 2 ml of 30% methanolic sulphuric acid were added (giving a sulphuric acid concentration of *ca.* 7.5 *M*). The tubes were heated at 90°C for 1, 2, 6 or 15 h.

In a further experiment, the effect of water on the rate of conversion was tested. For this, part of the

methanol (10% and 20%) was replaced with water. Methanolic hydrochloric acid was also used for methanolysis.

Methanolysis of erythrocyte membrane phospholipids separated by HPLC

Phospholipids separated by HPLC were collected, the solvent was evaporated and phospholipids were dissolved in 1 ml of chloroform–methanol (1:1). The phosphoglycerides were methanolysed with either methanolic sodium methoxide or boron trifluoride–methanol reagent. In the former instance, 4 ml of 0.5 *M* methanolic sodium methoxide solution were added to the test-tubes containing phosphoglyceride classes and the tube was shaken for 1 h. FAMEs were extracted twice by adding 2 ml each of *n*-hexane and water. The hexane phases were collected. In the latter instance, 2 ml of boron trifluoride–methanol reagent were added to the test-tubes, which were closed and heated at 90°C for 2 h. FAMEs were extracted twice by adding 0.5 ml each of *n*-hexane and water. Sphingomyelin was methanolysed using boron fluoride reagent. A 2-ml volume of boron trifluoride–methanol reagent was added to the test-tubes containing the sphingomyelin fraction. The tubes were closed and heated at 90°C for 15 h. FAMEs were extracted twice by adding 0.5 ml each of *n*-hexane and water.

Gas chromatographic analysis of FAMEs

FAMEs were analysed as described by Eder *et al.* [38]. A 0.5-μl portion of the FAME extract was injected manually into the GC system using a PTV system. For injection of FAMEs from phospholipid standards the splitting ratio was 1:20 and for injection of FAMEs from rat erythrocyte membranes it was 1:2. Hydrogen was used as the carrier gas at a flow-rate of 2.0 ml/min. The PTV programme was as follows: initial temperature, 40°C, held for 1 min, then increased at 800°C/min to 300°C, which was maintained for 10 min. The flame ionization detector temperature was 300°C. After an initial temperature of 50°C for 1 min, the oven temperature was increased at 25°C/min to 160°C, then at 15°C/min to 200°C, held at that temperature for 1.5 min, and subsequently increased at 10°C/min to 225°C, which was then maintained for a further 10 min. The amount of each FAME was calculated using C_{17} FAME as internal standard. As an example, the GC separation of FAMEs from phospholipids from egg is shown in Fig. 1.

Calculation of conversion rates

Conversion rates were calculated from the ratio between fatty acids converted into FAMEs and total phospholipid-bound fatty acids. The amounts of total phospholipid-bound fatty acids were calculated by determining the average molecular mass of the fatty acids, the molecular mass of the phospholipid core, the mass of total phospholipids and the amount (in percent) of fatty acids in the phospholipid molecule.

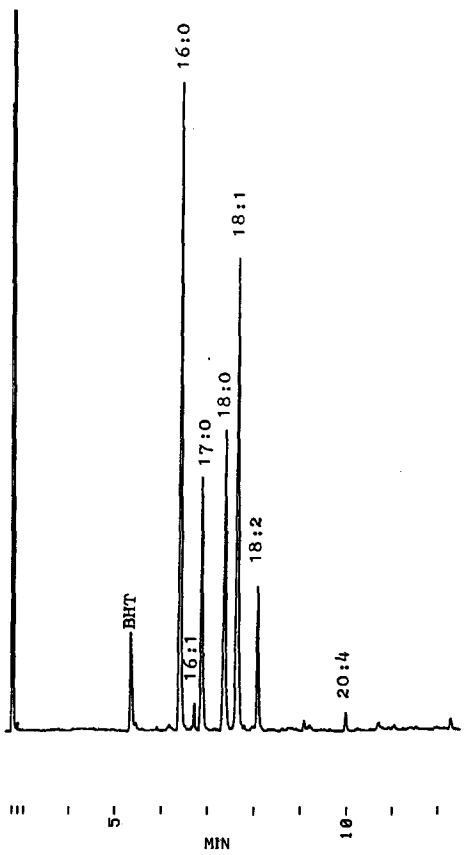

Fig. 1. Separation of fatty acid methyl esters from egg phospholipids obtained by methanolysis with methanolic sodium methoxide. Splitting ratio 1:20; 17:0 = internal standard; time scale in minutes.

RESULTS AND DISCUSSION

Methanolysis of phosphoglycerides

Various methods were applied to the methanolysis of small amounts of standard phospholipids and phospholipids from rat erythrocyte membranes separated by HPLC. In the first experiment, the rate of conversion of standard phospholipid bound fatty acids into FAMEs was checked using various methods based on methanolysis with (A) boron trifluoride–methanol (90°C, 30 min), (B) methanolic sodium methoxide (75°C, 20 min), (C) methanolic sodium methoxide (ambient temperature, 60 min) and (D) methanolic sulphuric acid (90°C, 2 h). Method E was based on saponification with methanolic sodium hydroxide and subsequent esterification with boron trifluoride–methanol. Rates of phospholipid-bound fatty acids converted into FAMEs using various methods are shown in Table I.

Of the methods investigated, only reaction with sodium methoxide at ambient temperature gave results that could be considered to be reasonable. For the other four methods, the yields of FAMEs from individual phospholipid classes were so variable and so lacking in reproducibility that they cannot be considered to be viable quantitative methods. Even in the case of reaction with sodium methoxide at ambient temperature, the yields were scattered to the extent that it would appear that there is still the need to improve control over the experimental conditions. Table II shows that methods A, B, D and E gave lower rates of conversion of both saturated and unsaturated fatty acids into FAMEs than the method based on sodium methoxide at ambient temperature. This indicates that the low yields of FAMEs produced by methods A, B, D and E are not due to oxidation of polyunsaturated fatty acids during heating, but to unreasonable experimental conditions in general. Similar results were obtained in the methanolysis of phospholipids from egg. The method based on sodium methoxide at ambient temperature gave the highest yields of FAMEs, whereas the yields of FAMEs obtained by methanolysis using the boron trifluoride method were smaller (Table III).

In a further experiment, phospholipids from rat erythrocyte membranes separated by HPLC were methanolysed using either methanolic sodium methoxide at ambient temperature for 1 h or boron

TABLE I

RATES OF PHOSPHOLIPID-BOUND FATTY ACIDS CONVERTED INTO FAMEs USING VARIOUS METHODS

Results are given in means ($n = 3$) and ranges (%)

Pl class[a]	Method				
	A[b]	B[c]	C[d]	D[e]	E[f]
PC	88 (86–89)	96 (96–97)	99 (98–100)	79 (71–86)	91 (90–91)
PE	84 (81–87)	99 (98–101)	103 (101–106)	82 (81–83)	98 (94–101)
PS	70 (64–75)	102 (100–103)	97 (96–98)	74 (68–82)	85 (84–86)
LPC	83 (82–86)	93 (93–94)	98 (96–99)	80 (79–82)	83 (81–84)
LPE	91 (89–92)	99 (97–101)	101 (100–102)	91 (90–92)	90 (90–91)

[a] Phospholipid classes in amounts of 0.5–1 mg.
[b] A: boron trifluoride–methanol (30 min at 90°C).
[c] B: 0.5 *M* methanolic sodium methoxide (20 min at 75°C).
[d] C: 0.5 *M* methanolic sodium methoxide (1 h at ambient temperature).
[e] D: 6% methanolic sulphuric acid (2 h at 90°C).
[f] E: saponification with subsequent esterification.

TABLE II

AMOUNTS OF THE MOST IMPORTANT PHOSPHOLIPID-BOUND FATTY ACIDS CONVERTED INTO FAMEs USING VARIOUS METHODS

Results are mean (n = 3) μg FAMEs per mg of phospholipid.

Pl class	Fatty acid	Method				
		A[b]	B[c]	C[d]	D[e]	E[f]
PC	16:0	196	218	231	185	205
	18:0	353	423	438	350	402
	20:0	28	34	32	25	35
PE	16:0	617	729	759	597	716
PS	18:0	239	335	319	255	286
	18:1	170	231	218	177	192
	22:6	36	82	75	48	68
LPC	16:0	288	322	334	280	302
	18:0	118	135	145	112	102
	18:1	16	18	19	15	14
LPE	16:0	208	218	218	214	190
	18:0	298	336	345	290	310

[b–f] See Table I.

trifluoride–methanol at 90°C for 2 h. Both methods gave similar yields of fatty acids converted into FAMEs (Table IV). This result, showing that for methanolysis of erythrocyte membrane phospholipids the boron trifluoride method is equivalent to the sodium methoxide method, contrasts with the results of methanolysis of standard phosphoglycerides, showing that the sodium methoxide method is superior to the boron trifluoride method. The reason for this inconsistency might be that both methods used for methanolysis of standard phospholipids were modified in the experimental conditions for methanolysis of erythrocyte membrane phospholipids. Using the boron trifluoride method the period of heating used was 30 min for methanolysis of standard phospholipids whereas it was 2 h for methanolysis of erythrocyte membrane phospholipids. Hence it seems that 30 min are not sufficient for completion of methanolysis of phospholipids using boron trifluoride at 90°C. This result is in contrast to the results of Morrison and Smith [7], which showed that phosphoglycerides can be methanolysed completely at 100°C in 10 min. Surprisingly, methanolysis with methanolic sulphuric acid under conditions often used [39,40] also gave incomplete conversion of fatty acids from standard phospholipids into FAMEs. The incompleteness of methanolysis might be due to the period of heating, as Freedman *et al.* [41] found that complete methanolysis of vegetable oils using 1% methanolic sulphuric acid takes over 50 h at 65°C. Saponification with sodium hydroxide and subsequent esterification with boron trifluoride–methanol also gave incomplete methanolysis. In this instance the incompleteness might be due to the short period of saponification (15 min) and might not be due to the short period of esterification (15 min), as Morrison and

TABLE III

METHANOLYSIS OF PHOSPHOLIPIDS FROM EGG USING VARIOUS METHODS

Results are mean μg fatty acids converted into FAMEs per mg of phospholipids from egg, with ranges.

FAME	Method		
	A[a] (n=4)	B[b] (n=3)	C[c] (n=3)
16:0	187 (185–193)	264 (255–269)	258 (255–261)
16:1	9 (7–10)	11 (7–14)	12 (11–13)
18:0	73 (71–78)	94 (92–95)	107 (105–110)
18:1	125 (119–131)	173 (169–178)	185 (183–187)
18:2	46 (43–50)	62 (60–64)	65 (64–67)
20:4	6 (6–7)	8 (6–10)	9 (9–10)
Sum	447 (437–459)	611 (597–620)	636 (633–638)

[a] A: boron trifluoride–methanol (30 min at 90°C).
[b] B: 0.5 *M* methanolic sodium methoxide (20 min at 75°C).
[c] C: 0.5 *M* methanolic sodium methoxide (1 h at ambient temperature).

TABLE IV

METHANOLYSIS OF RAT ERYTHROCYTE MEMBRANE PHOSPHOLIPID CLASSES USING EITHER BORON TRIFLUORIDE OR SODIUM METHOXIDE

Results are μg phospholipid-bound fatty acids converted into FAMEs per 170 μl of packed erythrocytes (duplicate results).

Reagent	Sample No.	Phospholipid class				
		PS	PE	PE plasmalogens	PC	Sum
$NaOCH_3$[a]	1	44.2	58.7	51.8	192.1	346.8
	2	45.0	63.1	52.5	196.6	357.1
BF_3[b]	1	50.0	65.1	48.4	194.5	358.0
	2	47.6	61.7	51.7	189.0	350.0

[a] Ambient temperature, 1 h.
[b] 90°C, 2 h.

Smith [7] found that esterification of free fatty acids using boron fluoride is complete within 2 min. On the other hand, there are studies showing that phospholipids cannot be saponified completely [27].

Although in this study methods A, C and D did not give complete methanolysis of phosphoglyceride standards, it should be noted that this does not mean that the reagents used are not useful for methanolysis but rather that the experimental conditions used in this study did not allow complete methanolysis. In fact, many studies have shown that methods using boron trifluoride–methanol [7,8,27,28] or methanolic sulphuric acid [14,27], as well as the saponification–esterification procedure [26,42], are suitable for the complete methanolysis of glycerides, provided that the reaction conditions are optimized.

The results using standard phospholipids indicate that methanolic sodium methoxide might be the most useful reagent for the methanolysis of phospholipids in small amounts. Many investigators have also shown that methanolic sodium methoxide might be more useful for the methanolysis of fats and oils than the widely used boron fluoride and other reagents [17]. On the other hand, in this study methanolysis of erythrocyte membrane phosphoglycerides with boron trifluoride–methanol reagent for 2 h gave similar yields of FAMEs to those obtained by methanolysis with sodium methoxide. However, methanolysis with sodium methoxide is faster and simpler than methanolysis with boron trifluoride and can be applied at ambient temperature, so that simple tubes such as centrifuge tubes can be used. This offers the possibility of the use of a one-vial procedure. In the method described, phospholipid classes were separated by HPLC. The eluate containing phospholipid classes was collected in 10-ml centrifuge tubes and the solvent was evaporated under vacuum at ambient temperature. Then methanolysis of phospholipids and extraction of FAMEs were carried out using the same tubes. Hence losses of phospholipids during transfer from one tube to another can be completely avoided. Therefore, the recoveries of phospholipids were *ca.* 100% (98.9% for PE, 101.8% for PC). Moreover, methanolysis at ambient temperature did not require BHT. This is undoubtedly advantageous because in some instances, in the GC analysis of FAMEs, methyl myristate or palmitoleate and BHT and its derivatives cannot be separated [43]. In Fig. 2 a typical GC separation of FAMEs from rat erythrocyte membrane PE obtained by methanolysis with the method described is shown.

Methanolysis of plasmalogens

The major phospholipids, namely PC and PE, exist in plasmalogen and non-plasmalogen subfractions [43,44]. In this study, a PE standard from bovine brain which contains *ca.* 60% plasmalogen was methanolysed using boron trifluoride–methanol reagent, methanolic sulphuric acid and methanolic sodium methoxide. Methanolysis of PE plasmalogen with boron trifluoride–methanol and methanol-

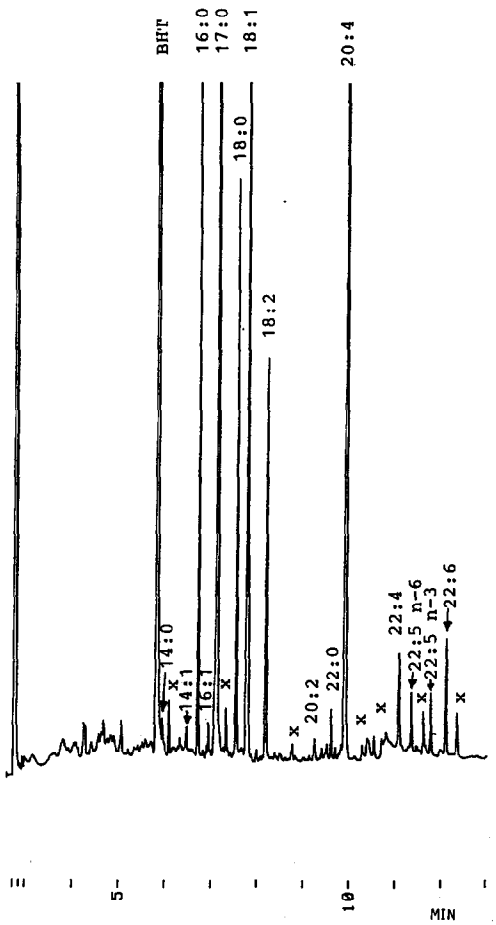

Fig. 2. Separation of fatty acid methyl esters from rat erythrocyte membrane phosphatidylethanolamine obtained by methanolysis with methanolic sodium methoxide. Splitting ratio 1:2; 17:0 = internal standard; time scale in minutes; x = unidentified peak.

ic sulphuric acid resulted in the formation of dimethylacetals (DMAs), which interfered with the GC separation of FAMEs (Fig. 3a and b). Using the conditions described, peaks of FAME 17:0 and DMA 18:0 could not be separated. However, the formation of DMAs was prevented if PE plasmalogen was methanolysed with sodium methoxide (Fig. 3c). This observation is in agreement with the results of other studies [45], also showing that DMA formation from plasmalogen aldehydes can be prevented by base-catalysed methanolysis. Hence, methanolic sodium methoxide is a useful reagent for the methanolysis of plasmalogens.

Another possibility for preventing DMA formation from plasmalogens is to hydrolyse the labile enol–ether binding prior to methanolysis by treatment of plasmalogens with concentrated HCl fumes [5] or 90% acetic acid [36,43]. If plasmalogens are injected into an HPLC system using a mobile phase containing phosphoric or sulphuric acid they are also hydrolysed during their passage through the column [5,34–36]. Thus plasmalogens elute as their 2-acyl lyso analogues. In this study, phospholipids from rat erythrocyte membranes were separated using a mobile phase containing 0.2% of orthophosphoric acid (85%). GC determination of the methylated fatty acids showed that the lyso-PE fraction eluted corresponds to the PE plasmalogen injected into the HPLC system as there is a large portion of polyunsaturated fatty acids with 20 and 22 carbon atoms which is typical of erythrocyte plasmalogens [43] (Fig. 4). Moreover, the chromatogram shows that there were no DMAs formed during methanolysis with boron trifluoride–methanol reagent.

Methanolysis of sphingomyelin

Sphingomyelin is extremely resistant to alkali treatment because fatty acids exist as amides rather than as esters. Therefore, methanolic inorganic acids such as hydrochloric and sulphuric acid and also boron trifluoride–methanol are the most often used catalysts for the methanolysis of sphingomyelin [7,46–49]. However, in the literature there is little information about experimental conditions that ensure complete methanolysis of sphingomyelin. Moreover, the published results are not consistent. For example, Morrison and Smith [7] found that when using boron trifluoride–methanol, methanolysis of sphingomyelin is complete within 75 min. In studies by MacGee and Williams [50], the rate of conversion of fatty acids from sphingomyelin into FAMEs using boron trifluoride–methanol reagent was only 25% within 2 h. Therefore, in this study, methanolysis was carried out using boron trifluoride and methanolic sulphuric acid in order to investigate the effect of the period of heating on the rates of conversion of sphingomyelin fatty acids. Our results are in agreement with those of MacGee

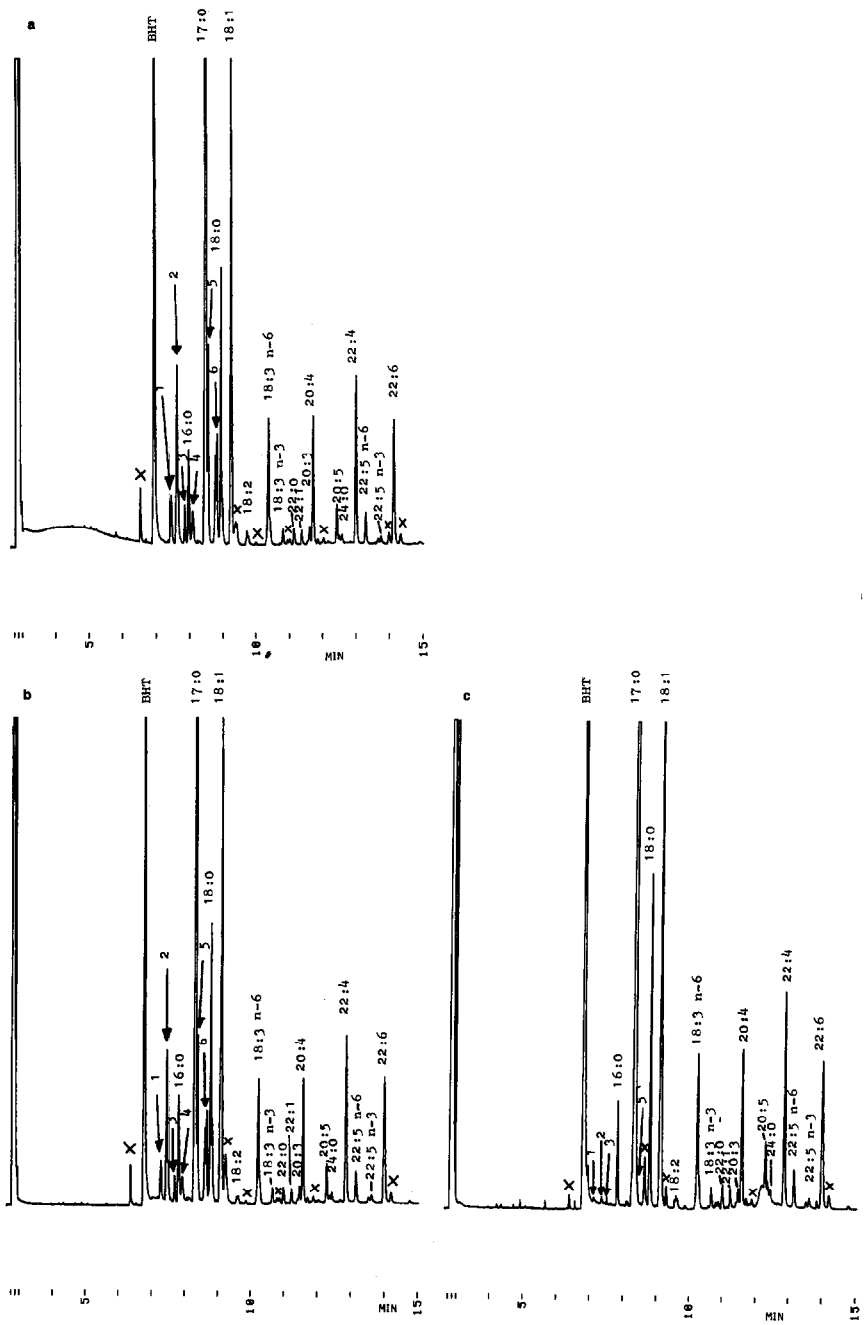

Fig. 3. Separation of fatty acid methyl esters from bovine brain phosphatidylethanolamine containing 60% plasmalogens. Fatty acid methyl esters were prepared using (a) boron trifluoride–methanol reagent, (b) methanolic sulphuric acid and (c) methanolic sodium methoxide. Arrows indicate dimethylacetals formed during methanolysis; 17:0 = internal standard; time scale in minutes; x = unidentified peak.

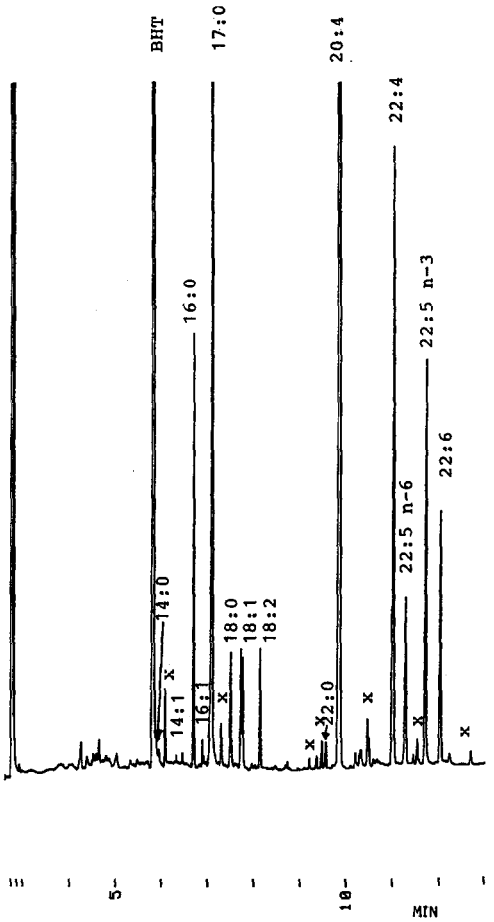

Fig. 4. Separation of fatty acid methyl esters from rat erythrocyte membrane phosphatidylethanolamine plasmalogen obtained by methanolysis with boron trifluoride–methanol reagent. Splitting ratio = 1:2; 17:0 = internal standard; time scale in minutes; x = unidentified peak.

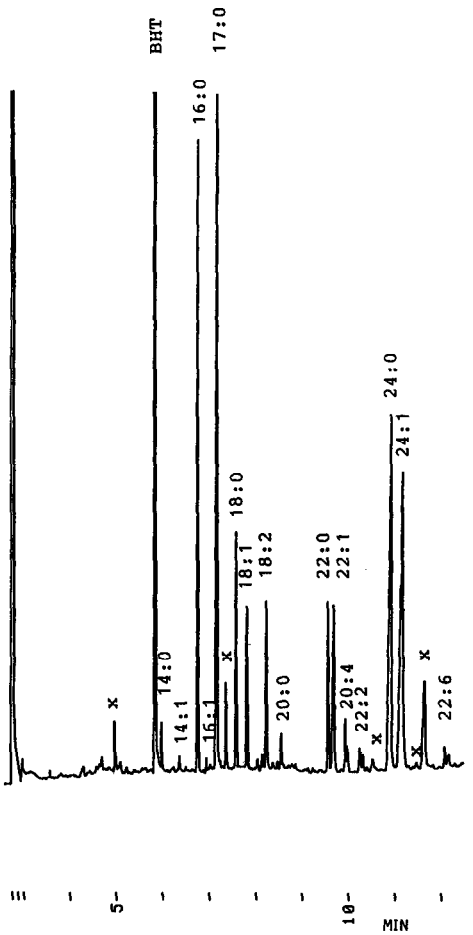

Fig. 5. Separation of fatty acid methyl esters from rat erythrocyte membrane sphingomyelin obtained by methanolysis with boron trifluoride–methanol reagent. Splitting ratio = 1:2; 17:0 = internal standard; time scale in minutes; x = unidentified peak.

and Williams [50], showing that very long periods of heating are necessary for complete conversion of sphingomyelin fatty acids into FAMEs using both boron trifluoride and methanolic sulphuric acid (1 and 2.5 *M*) (Table V).

Using methanolic sulphuric acid and boron trifluoride–methanol, complete methanolysis was achieved after heating at 90°C over a period of 15 h. However, the rates of conversion for periods of 1, 2 and 6 h were higher for methanolic sulphuric acid than for boron trifluoride. Moreover, the period of heating required for complete methanolysis could not be shortened by increasing the concentration of sulphuric acid. Using 7.5 *M* methanolic sulphuric acid, the rate of conversion decreased with an increase in heating time. This phenomenon is due to oxidation of unsaturated fatty acids caused by high temperature and high concentration of sulphuric acid.

Although a large amount of BHT (100 μg) was added to each test-tube, the amounts of polyunsaturated FAMEs decreased with an increase in heating time (Table VI). On the other hand, the fatty acid composition of sphingomyelin standard ob-

TABLE V

EFFECT OF PERIOD OF HEATING ON RATES OF CONVERSION OF SPHINGOMYELIN FATTY ACIDS INTO FAMEs USING BORON TRIFLUORIDE OR METHANOLIC SULPHURIC ACIDS AT VARIOUS CONCENTRATIONS

Results are means ($n=3$) and ranges (%)

Reagent	Period of heating (h)			
	1	2	6	15
BF_3	35.5 (32.6–37.3)	60.4 (58.1–63.1)	82.7 (82.0–83.2)	99.1 (98.6–99.4)
1.0 *M* H_2SO_4	53.5 (52.7–54.2)	81.4 (79.4–83.0)	94.6 (93.0–96.0)	97.6 (96.3–99.6)
2.5 *M* H_2SO_4	75.5 (73.9–76.9)	87.6 (86.4–89.0)	92.4 (90.2–94.8)	100.3 (96.0–103.4)
7.5 *M* H_2SO_4	74.8 (73.5–76.2)	84.8 (83.6–85.5)	73.1 (71.5–74.4)	65.6 (64.8–66.2)

tained by methanolysis with boron trifluoride–methanol reagent and using various periods of heating was similar (Table VII). Moreover, the fatty acid composition of sphingomyelin standard obtained by methanolysis was similar using 1 and 2.5 *M* methanolic sulphuric acid and boron trifluoride (Table VIII).

Methanolic hydrochloric acid is also often used for the methanolysis of sphingomyelin [46–48]. In the present experiments, the rates of conversion of sphingomyelin fatty acids into FAMEs using 2.4 *M* methanolic hydrochloric acid and 2.25 *M* methanolic sulphuric acid were similar. Moreover, the addition of small amounts of water (200–400 μl, representing 10–20% of the total volume) did not influence the rate of conversion. On adding 0, 10 and 20% of water, the rates of conversion of sphingomyelin standard fatty acids into FAMEs using 2.25 *M* methanolic sulphuric acid were 97.9, 98.9 and 97.1% respectively, and using 2.4 *M* methanolic hydrochloric they were 99.9, 100.8 and 101.7% respectively. This indicates that methanolic acids are also useful for the methanolysis of sphingomyelin from extracts containing small amounts of water. However, 10% or more of water disturbs the methanolysis of sphingomyelin using methanolic acetyl chloride [22].

The method for the determination of fatty acid composition of sphingomyelin from rat erythrocyte membranes described used boron trifluoride as catalyst. The separation of FAMEs from rat erythrocyte membrane sphingomyelin obtained by this method is shown in Fig. 5.

TABLE VI

CONVERSION OF SPHINGOMYELIN FATTY ACIDS INTO FAMEs USING 7.5 *M* SULPHURIC ACID AND VARIOUS PERIODS OF HEATING

Results are mean μg sphingomyelin-bound fatty acids converted into FAMEs per mg of sphingomyelin ($n=3$).

Fatty acid	Period of heating (h)			
	1	2	6	15
16:0	10.1	11.8	12.6	12.6
18:0	113.3	136.9	144.3	134.7
20:0	3.6	3.7	5.5	5.3
22:0	12.9	15.2	16.0	15.0
24:0	30.2	36.6	35.0	38.2
20:4	21.0	24.9	19.9	13.4
22:1	2.9	2.9	3.0	2.3
22:2	6.3	7.4	4.2	1.9
24:1	110.1	109.2	51.5	37.2

CONCLUSION

For the methanolysis of small amounts of phosphoglycerides, sodium methoxide is the most useful reagent. Sodium methoxide methanolysis of phosphoglycerides from rat erythrocyte membranes, separated by HPLC, can be carried out at ambient temperature using a one-vial procedure. This meth-

TABLE VII

FATTY ACID COMPOSITION OF SPHINGOMYELIN STANDARD OBTAINED BY METHANOLYSIS WITH BORON TRIFLUORIDE USING VARIOUS PERIODS OF HEATING

Results are mean mol% ($n=3$) and ranges

Fatty acid	Period of heating (h)			
	1	2	6	15
16:0	4.6 (4.2–5.0)	4.7 (4.6–4.8)	4.6 (4.3–5.0)	3.9 (3.6–4.1)
18:0	43.1 (41.4–44.7)	44.5 (41.8–47.6)	41.1 (40.7–41.5)	42.7 (41.0–43.9)
20:0	1.3 (0.6–1.7)	0.9 (0.8–0.9)	1.1 (0.9–1.3)	0.9 (0.8–1.0)
20:4	7.4 (7.1–7.9)	7.4 (6.7–8.0)	8.9 (8.6–9.3)	7.6 (7.2–8.0)
22:0	3.6 (3.4–3.9)	3.9 (3.5–4.2)	4.3 (3.8–4.7)	3.7 (3.3–4.0)
22:1	1.2 (0.5–1.8)	1.1 (0.9–1.3)	1.2 (1.0–1.4)	0.9 (0.6–1.2)
22:2	1.8 (1.7–2.0)	1.7 (1.5–2.0)	1.3 (1.2–1.3)	1.9 (1.8–2.0)
24:0	6.7 (6.0–7.2)	6.3 (6.0–6.6)	6.8 (6.7–7.0)	7.9 (7.5–8.1)
24:1	30.3 (25.4–35.6)	29.5 (25.7–31.6)	30.7 (29.1–32.1)	30.5 (30.0–31.5)

TABLE VIII

FATTY ACID COMPOSITION OF SPHINGOMYELIN STANDARD OBTAINED BY METHANOLYSIS WITH BORON TRIFLUORIDE AND 1 AND 2.5 *M* METHANOLIC SULPHURIC ACID

Period of heating 15 h in each instance. Results are mean mol% ($n=3$) and ranges

Fatty acid	Reagent for methanolysis		
	BF_3	1 *M* H_2SO_4	2.5 *M* H_2SO_4
16:0	3.9 (3.6–4.1)	4.3 (4.1–4.6)	4.2 (4.2–4.4)
18:0	42.7 (41.0–43.9)	42.2 (41.9–42.4)	41.5 (40.4–43.4)
20:0	0.9 (0.8–1.0)	0.9 (0.9–1.0)	0.9 (0.8–1.0)
20:4	7.6 (7.2–8.0)	6.8 (5.3–8.4)	7.5 (6.5–9.1)
22:0	3.7 (3.3–4.0)	3.9 (3.7–4.3)	4.3 (4.3–4.5)
22:1	0.9 (0.6–1.2)	1.0 (0.8–1.0)	0.9 (0.9–1.1)
22:2	1.9 (1.8–2.0)	1.5 (1.4–1.6)	1.3 (1.1–1.3)
24:0	7.9 (7.5–8.1)	8.0 (7.8–8.4)	9.0 (8.4–10.0)
24:1	30.5 (30.0–31.5)	31.4 (30.8–32.4)	30.4 (29.1–32.1)

od is simple and gives complete methanolysis, as was demonstrated by using phospholipid standards. For the methanolysis of sphingomyelin, boron trifluoride and also methanolic sulphuric and hydrochloric acid can be used. The study presented shows that complete methanolysis of sphingomyelin takes about 15 h.

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