

Quantitative release of fatty acids from lipids by a simple hydrolysis procedure

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Summary Glycerolipids and sphingolipids are hydrolyzed with 0.5 M HCl in acetonitrile–water 9:1 (by vol) for 45 min at 100°C or 4 hr at 70°C. After hydrolysis, free fatty acids (FFA) are recovered in chloroform and separated by thin-layer chromatography. More than 95% of the radioactivity from labeled phospholipids is recovered as FFA, and more than 97% of the lipid phosphorus is recovered as water-soluble phosphate. The yields of FFA, including polyunsaturated acids, after hydrolysis are as good as or better than those obtained for methyl esters using methanolysis catalyzed by acid, alkali, or BF₃. High recoveries of FFA from glycerophospholipids, sphingomyelin, and neutral glycerides are attained. The procedure is quantitative, simple, inexpensive, and produces no methyl esters as secondary products.—**Aveldaño, M. I., and L. A. Horrocks.** Quantitative release of fatty acids from lipids by a simple hydrolysis procedure. *J. Lipid Res.* 1983. **24:** 1101–1105.

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High performance liquid chromatographic (HPLC) procedures for the analysis of free fatty acids or methyl esters have recently been described (1). HPLC separations can be used to determine specific radioactivities of individual fatty acids from lipids in studies on turnover and metabolic conversions of fatty acids. However, FFA are preferable to FAME for this purpose since the separations can be achieved in less time. FFA are also required for the preparation of other derivatives for HPLC separations.

Acid-, alkali-, or boron halide-catalyzed methanolysis procedures are extensively used to prepare methyl esters from lipids, particularly for gas–liquid chromatography. BF₃-methanol has been recommended to produce methyl esters from total lipid extracts, since mixtures containing glycerophospholipids, sphingolipids, and neutral lipids can be converted to methyl esters in about 90 min at 100°C (2). However, the production of free fatty acids for analytical purposes is not widely used. The strong acid or alkaline conditions that are

required to release fatty acids from sphingolipids may give rise to side reactions or structural changes in other fatty acids present in the samples. Esterified fatty acids can be released at room temperature by saponification in the presence of an alcohol. This procedure is gentle enough to preserve the integrity of polyunsaturated fatty acids, but does not release amide-bound fatty acids, and variable amounts of methyl (or ethyl) esters are produced due to the presence of the alcohol used as solvent.

In this report a simple and quantitative procedure using HCl in acetonitrile is shown to hydrolyze lipids containing ester or amide bonds, yielding FFA that can be analyzed by HPLC (1). HCl in acetonitrile has also been used to release long chain bases from gangliosides without the formation of O-methyl derivatives (3).

MATERIALS AND METHODS

Lipid sources and preparations

Labeled phospholipids were prepared after intraventricular injections of [1-¹⁴C]acetate into mouse brains. Lipids were extracted (4) and total phospholipids were isolated by silicic acid column chromatography (5). Unlabeled glycerophospholipids were isolated from bovine brain lipid extracts by TLC, using magnesium acetate-containing silica gel H plates (Redicoat, Supelco, Bellefonte, PA), 400 μm, developed with chloroform–methanol–ammonia 13.5:5:1 (by vol). Major glycerophospholipids, except sphingomyelin, were eluted (6) and combined. Sphingomyelin, commercial lecithins, trioleoylglycerol, and dipalmitoylglycerol were purchased from Sigma Chemical Co. (St. Louis, MO).

Hydrolysis of lipids and recovery of free fatty acids

Acetonitrile (Burdick and Jackson, Muskegon, MI or MCB Manufacturing Chemists, Cincinnati, OH) and water were thoroughly degassed and bubbled with nitrogen before using. A 0.5-M solution of HCl was freshly prepared in acetonitrile–water 9:1 (by vol), and kept under nitrogen. Lipid aliquots were taken to dryness under N₂ in screw-capped tubes and 1 ml of HCl–CH₃CN solution was added. After flushing with N₂, the tubes were closed with Teflon-lined caps. Hydrolysis was performed in a water bath at 100°C without agitation or in a stirring-heating module (Pierce Chemical Company, Rockford, IL) at 70°C, with magnetic agitation. After hydrolysis, 1 ml of CH₃Cl and 1 ml of water were added, the solvents were thoroughly mixed, and the phases were separated by centrifugation. A backwash with 1 ml of water was also performed. The organic phase was dried under N₂ and the lipids were

Abbreviations: HPLC, high performance liquid chromatography; GLC, gas–liquid chromatography; FFA, free fatty acids; FAME, fatty acid methyl esters; 20:4, arachidonic acid.

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separated by TLC on silica gel G plates (Analtech, Newark, DE), 250 μm , using hexane–ethyl ether–acetic acid 80:20:1.5 (by vol). The TLC step is necessary when FFA are prepared from total lipid extracts to remove cholesterol and other nonhydrolyzable lipids, as well as other products of hydrolysis such as sphingomyelin and aldehydes. The position of unlabeled FFA was ascertained by exposing standards, located at both sides of the plates, to iodine vapors. FFA were eluted by three successive extractions with 4 ml of HPLC quality chloroform–methanol–acetic acid–water 50:39:1:10 (by vol) (6), followed by one partition with 4 ml of water. Labeled FFA were located directly with iodine vapors and either eluted as above or scraped into vials for liquid scintillation counting.

Preparation of methyl esters

Alkaline methanolysis was done by adding 1 ml of 0.5 M NaOH in degassed methanol, and stirring the samples under N_2 at 25°C for 30 min. After adding 1 ml of water, three extractions into 3 ml of hexane were done. Acid-catalyzed methanolysis was performed with 1 ml of 0.7 M HCl in degassed methanol (prepared from acetyl chloride, Applied Science Labs., State College, PA) for 60 min at 100°C. This was followed by addition of water and extraction into hexane as above. BF_3 -catalyzed methanolysis was done according to the procedure of Morrison and Smith (2) using 14% BF_3 in methanol (Regis, Chicago, IL). Free fatty acids produced by HCl-acetonitrile hydrolysis were methylated using ethereal diazomethane (freshly prepared from N-methyl-N'-nitro-N-nitrosoguanidine, Aldrich Chemical Co., Milwaukee, WI). After direct methanolysis or hydrolysis-methylation, the methyl ester of 21:0 was added as internal standard for GLC quantitation, and all methyl ester preparations were subjected to TLC using 250 μm silica gel G plates and benzene (2). The plates were sprayed with dichlorofluorescein and esters were isolated with the solvents described by Arvidson (6), including a partition with 4 M NH_4OH to eliminate the dye.

Gas–liquid chromatography

A Model 428 gas chromatograph (Packard, Downers Grove, IL) equipped with a glass column packed with 10% CS-TO on Chrom WAW (Aldrich) was used. Injector, column, and flame ionization detector temperatures were 220°, 190°, and 220°C, respectively. N_2 carrier gas flowrate was 30 ml/min. Areas were determined using a System I computing integrator (Spectra-Physics, Santa Clara, CA).

Liquid scintillation counting

Scrapings from TLC plates containing labeled FFA were transferred to vials, suspended in 1 ml of water

(7), and mixed with 10 ml of Readi-Solv (Beckman Instruments, Berkeley, CA). Aliquots from eluted FFA were taken to dryness and counted with 10 ml of Readi-Solv. An LS 7000 liquid scintillation counter (Beckman) was used. Counting efficiencies were determined using ^3H or ^{14}C toluene standards (New England Nuclear, Boston, MA).

Phosphorus analysis

Aliquots from phospholipid solutions and from the aqueous phases after hydrolysis were taken to dryness, and phosphate was measured colorimetrically after digestion with HClO_4 (8).

RESULTS

A ^{14}C -labeled sample of total phospholipids obtained from mouse brain was used to determine the time-course for the release of fatty acids using HCl-acetonitrile. Aliquots containing 1 μg of P were hydrolyzed for periods up to 90 min at 100°C and up to 300 min at 70°C. After hydrolysis, the lipids were extracted, washed, and separated by TLC. The phospholipid and FFA spots were scraped into vials and counted in the presence of silica gel. More than 97% of the radioactivity originally associated with phospholipids was recovered as FFA from 30 min and 120 min onwards at 100° and 70°C, respectively. To ensure completion of hydrolysis when larger amounts of lipids were used, 45 min at 100°C or 240 min at 70°C were used in the experiments described later.

HCl-acetonitrile also produced a rapid release of phosphate, recovered in the aqueous phase, when 5 mg of a commercial preparation of egg yolk phosphatidylcholine was used (Fig. 1, insets). The yield of water-soluble phosphate was proportional to the amount of lipid in the range of 1 to 40 mg (0.038 to 1.5 mg of lipid P) (Fig. 1). The recovery of P in the aqueous phase from 40 mg of lipid was 99.2 and 95.9% of the P originally present in the organic phase, after 45 min at 100°C and 240 min at 70°C, respectively. The same conditions gave 98 and 96% recovery of water-soluble phosphate from 1 to 4 mg of sphingomyelin. The nature of water-soluble phosphates released after HCl-acetonitrile treatment was not ascertained. The quantitative recovery of P indicates that HCl- CH_3CN effectively hydrolyzes ester bonds in a wide range of phospholipid concentrations.

The effect of temperature on the recovery of polyunsaturated fatty acids was tested by heating ^{14}C - or ^3H -labeled arachidonic acid (oleic acid was added as carrier) for 240 min at 70°C or 45 min at 100°C. Heated samples as well as unheated controls (in quintuplicate) were subjected to partition and TLC, and FFA were counted

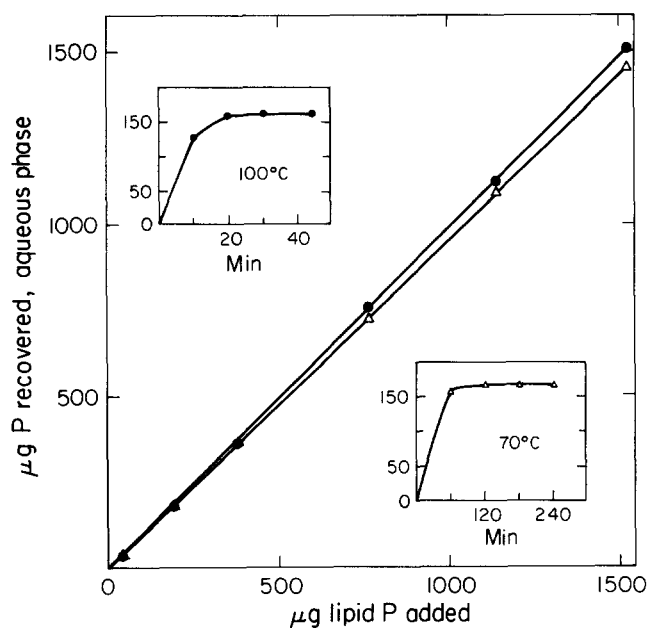


Fig. 1. Recovery of phosphorus in the aqueous phase after hydrolysis of egg yolk phosphatidylcholine (commercial grade) with 1 ml of 0.5 M HCl in acetonitrile. Aliquots containing 1 to 40 mg were hydrolyzed for 45 min at 100°C (●) or 240 min at 70°C (Δ). Phosphorus was measured before hydrolysis and in the aqueous phase after hydrolysis and partition with chloroform-water. Slopes of the lines are 0.959 for hydrolysis at 70°C for 240 min and 0.993 for hydrolysis at 100°C for 45 min. In the inset the time-course for the hydrolysis of about 5 μg of the same lipid is shown. Each point is the average of two determinations.

in the presence of silica gel. Recoveries were 82.0 ± 0.5 and $81.2 \pm 0.8 \times 10^3$ dpm of ^{14}C -labeled 20:4 after heating at 70°C or 100°C, respectively, compared with $83.3 \pm 0.8 \times 10^3$ dpm in unheated samples. With ^3H -labeled 20:4 the recovery was slightly lower: 48.7 ± 0.7 and $47.7 \pm 0.8 \times 10^3$ dpm were obtained at 70°C and 100°C, respectively, compared with $50.2 \pm 0.7 \times 10^3$

for controls. Thus, the loss of radioactivity due to heating was higher at 100° than at 70°C, and higher for ^3H -labeled 20:4 than for ^{14}C -labeled 20:4. Part of the lost ^3H was recovered in the aqueous phase (1240 ± 170 dpm at 70°C and 1540 ± 70 dpm at 100°C, in contrast with only 360 ± 40 dpm in the unheated samples). Nevertheless, more than 97% of the ^{14}C and 95% of the ^3H was recovered as FFA after heating 45 min at 100°C.

The recovery of FFA after other steps of the procedure was also tested using [^{14}C]arachidonic acid. All of the radioactivity in FFA remained in the organic phase after partition. The organic phase could be back-washed with water without losing fatty acids. (Partition and washing eliminate most of the HCl as well as organic-insoluble crystalline material that forms upon heating of the solvents). After TLC, radioactivity in FFA eluted from the silica was 99% of that present in samples directly counted on the silica gel scrapings.

To test the recovery of lipid-bound fatty acids as FFA after the whole procedure, samples containing 3670 ± 21 and 35290 ± 436 dpm of ^{14}C -labeled phospholipids from mouse brain (four samples each, about 10 and 100 mg of P) were treated for 45 min at 100°C. After partition, TLC, and elution, 3550 ± 42 and 33680 ± 670 dpm, respectively, were recovered as FFA. Thus, the overall recoveries were higher than 95% for samples containing up to 2.5 mg of phospholipids.

The yield of FFA after hydrolysis with HCl- CH_3CN is compared with the yield of methyl esters after methanolysis in **Table 1**. A glycerophospholipid preparation was subjected to acid, alkali, and BF_3 -catalyzed methanolysis to use the "best" method, in our hands, as a control for the hydrolysis procedure. Alkaline methanolysis yielded 9.0 to 9.6% less methyl esters than did acid methanolysis, with no significant differences in fatty acid composition. BF_3 -catalyzed methanolysis and acid

TABLE 1. Comparison of the yields of fatty acids after methanolysis and hydrolysis of bovine brain glycerophospholipids

Procedure	Fatty Acid Methyl Esters					Total ^a
	16:0	18:0	18:1	20:4	22:6	
	μg					
A. Methanolysis						
0.7 M HCl in CH_3OH , 50 min, 100°C	148 ± 2	105 ± 1	213 ± 2	43 ± 0.4	45 ± 0.4	619 ± 5
0.5 M NaOH in CH_3OH , 30 min, 25°C	140 ± 5 ^c	97 ± 2 ^b	205 ± 2 ^b	39 ± 0.4 ^b	43 ± 0.3 ^b	578 ± 12 ^b
14% BF_3 in CH_3OH , 10 min, 100°C	156 ± 7	108 ± 6	214 ± 11	42 ± 1.3	40 ± 1.3 ^b	627 ± 19
B. Hydrolysis						
0.5 M NaOH in 90% CH_3OH , 60 min, 70°C	150 ± 2	102 ± 2	213 ± 2	42 ± 0.4	45 ± 0.4	608 ± 6
0.5 M HCl in CH_3CN , 240 min, 70°C	152 ± 4	104 ± 2	212 ± 3	42 ± 0.6	44 ± 0.9	618 ± 8
0.5 M HCl in CH_3CN , 45 min, 100°C	151 ± 2	102 ± 3	210 ± 3	42 ± 0.6	44 ± 1.0	613 ± 6

Methyl esters or free fatty acids were obtained as indicated from aliquots of glycerophospholipids from bovine brain. The free fatty acids were converted to methyl esters with diazomethane. All samples were subjected to TLC after adding methyl 21:0 as internal standard. Methyl esters were eluted and quantitated by GLC. Values are means ± SD from five samples.

^a Includes all fatty acids present in the samples.

^{b,c} Significantly lower than values for the first procedure; *b*, $P < 0.01$; *c*, $P < 0.02$.

methanolysis gave similar yields, but the yield of 40 μg of 22:6 was significantly lower for the former. The amount of 22:6 was only $35.8 \pm 1.0 \mu\text{g}$ in samples heated with BF_3 -methanol for 95 min, which is the recommended procedure to methanolyze total lipid extracts (2). Thus, acid methanolysis that gave 45 μg of 22:6 was used as a control. The amounts of total and individual FFA obtained after hydrolysis with $\text{HCl-CH}_3\text{CN}$ were similar to those of the respective methyl esters after acid methanolysis. Moreover, the fatty acid composition after heating with $\text{HCl-CH}_3\text{CN}$ did not differ significantly from that obtained by the milder procedures used for alkaline hydrolysis or alkaline methanolysis. Therefore, the yields of FFA and fatty acid profiles after acid hydrolysis are as good as or better than those obtained for FAME after methanolysis.

To assess whether $\text{HCl-CH}_3\text{CN}$ can be used to hydrolyze fatty acids in amide linkage, sphingomyelin from bovine brain was subjected to hydrolysis at 100°C (Table 2). A 2- and 4-fold increase in hydrolysis time gave no further significant increase in the yield of FFA (98% of the amount obtained after 90 min). However, the slightly higher dispersion of the values obtained with 45 min of hydrolysis suggest that this may be the minimum time required at 100°C to hydrolyze sphingomyelin. About 10% more fatty acids were recovered by this procedure than by heating 90 min at 100°C with BF_3 -methanol, a procedure which was reported to give a 99% yield of methyl esters from sphingomyelin (2).

In Table 3 the amount of lipid that can be hydrolyzed per ml of reagent at 100°C for 45 min was tested by increasing 10 and 100 times the amount of a commercial preparation of soya bean lecithin (containing various phospholipid classes and some neutral lipids) and quantitating the FFA by GLC. A 9.8- and 95-fold increase in the yield of FFA was obtained. When an amount of sphingomyelin similar to that shown in Table 2 to be hydrolyzed by $\text{HCl-CH}_3\text{CN}$ was increased 10-fold, a 9.7-

TABLE 3. Capacity of HCl -acetonitrile to hydrolyze different types and amounts of lipids

Lipid	Approximate weight of lipid, mg ^a		
	0.5	5	50
Soya bean lecithin (commercial grade) ^b	298 ± 3^c	2914 ± 40	28340 ± 420
Sphingomyelin (bovine brain)	167 ± 3	1618 ± 64	
Trioleoylglycerol	510 ± 13	5450 ± 77	
Dipalmitoylglycerol	489 ± 3	4879 ± 120	

^a The indicated amounts of lipids were hydrolyzed with 1 ml of HCl in acetonitrile for 45 min at 100°C .

^b Contains 10–20% phosphatidylcholine and other lipids, including several phospholipids and neutral glycerides.

^c Values are μg fatty acid methyl esters \pm SD from three samples.

fold increase in the yield of fatty acids was obtained. About a 10-fold increase in the yield of FFA was obtained after increasing 10 times the amount of a tri- and a diacylglycerol, with a 99% recovery, at both concentrations, with respect to the theoretical value. Therefore, a lipid extract containing 50 mg of total lipid and up to 10% sphingomyelin and neutral glycerides could be hydrolyzed per ml of reagent with yields higher than 90%. However, these extremely concentrated solutions, used as a difficult test of the method, are unnecessary if the procedure is to be used to hydrolyze lipids from mammalian tissues. Amounts up to 5 mg total lipid per ml of reagent may be considered as "safe" concentrations for quantitative yields of FFA for most applications.

DISCUSSION

HCl in aqueous acetonitrile quantitatively releases fatty acids from glycerophospholipids, sphingomyelin,

TABLE 2. Yields of fatty acid methyl esters after methanolysis or hydrolysis-methylation of bovine brain sphingomyelin

Procedure	Time	Fatty Acid Methyl Esters				Total ^a
		18:0	24:0	24:1	26:1	
	min	μg				
A. Methanolysis						
14% BF_3 in CH_3OH	90	51.6 ± 0.6^c	8.8 ± 0.6^c	54.0 ± 0.7^b	6.3 ± 0.6	141.4 ± 1.0^b
B. Hydrolysis						
0.5 N HCl in 90% CH_3CN	45	55.7 ± 3.1	9.8 ± 0.2	58.8 ± 0.9	6.9 ± 0.3	155.3 ± 3.3
	90	55.8 ± 1.0	10.2 ± 0.2	61.7 ± 0.8	7.1 ± 0.6	158.0 ± 1.7
	180	56.4 ± 1.5	9.8 ± 0.2	60.6 ± 0.4	7.3 ± 0.2	157.4 ± 1.5

Methyl esters or free fatty acids were obtained by heating at 100°C as indicated. The free fatty acids were methylated with diazomethane, and methyl 21:0 was added to all samples. Esters were separated by TLC and quantitated by GLC. Values are means \pm SD from four samples.

^a Includes all fatty acids present in the samples.

^{b,c} Significantly lower than hydrolysis for 45 min; *b*, $P < 0.001$; *c*, $P < 0.05$.

and neutral glycerides, as well as phosphate from glycerophospholipids and sphingomyelin. This indicates that both ester and amide bonds are attacked by this reagent. The procedure can thus be applied to release FFA from total lipid extracts. After tissues or subcellular fractions are exposed to ^3H - or ^{14}C -labeled fatty acids, total lipid extracts can be hydrolyzed and the FFA can be separated by HPLC (1) to determine the distribution of radioactivity among precursors and products and the specific radioactivity of individual fatty acids.

Quantitative recoveries of polyunsaturated fatty acids from glycerophospholipids are obtained with the present procedure. Degassing and nitrogenation of the solvents before use is extremely important. Thus, significant amounts of 22:6 are lost in a time-dependent manner in samples heated at 100°C with 14% BF_3 in methanol, a reagent that is usually obtained commercially and obviously cannot be degassed before use.

Hydrolysis at 70°C is associated with lower losses of ^3H than those observed at 100°C . However, the latter permits shorter hydrolysis times with no significant differences in the yields of FFA, particularly polyunsaturates. In addition, solubility of lipids in the $\text{HCl}-\text{CH}_3\text{CN}$ mixture is higher at 100°C than at 70°C , which may facilitate hydrolysis. Heat-induced losses of radioactivity, probably due to accelerated radiolysis and/or ^3H exchange with protons, may be taken into account for determinations of specific activities of tritiated fatty acids, and the results can be corrected accordingly. However, since the losses, even at 100°C , are lower than 5%, uncorrected values should be reasonably accurate.

When distribution of radioactivity among fatty acids of a purified lipid is to be studied, for instance in choline or ethanolamine glycerophospholipids isolated by TLC, alkaline hydrolysis would be advantageous over the present procedure, particularly for ^3H -labeled samples. Thus, FFA can be released by leaving the samples a few hours in NaOH -aqueous methanol at room temperature, and after hydrolysis and acidification, virtually only FFA are recovered in a nonpolar solvent like hexane. A disadvantage of alkali-aqueous methanol with respect to acid-aqueous acetonitrile for lipid hydrolysis is that small amounts of methyl esters may be produced along with FFA with the former procedure. In HPLC separations of free fatty acids, some of these methyl esters may coelute with FFA, causing errors in mass measurements or, if labeled, in radioactivity determinations. These errors may be particularly serious when estimating percentage distribution of radioactivity

among fatty acids. Alkaline deacylation is also possible with monomethylamine, but the primary products from acyl groups are N-methyl fatty acid amides (9). Acyl groups are not hydrolyzed from sphingomyelin or other sphingolipids by any of the mild alkaline deacylation procedures.

The described procedure is simple, inexpensive, rapid, and quantitative. It can be used to release amide-bound fatty acids, which are quite resistant to hydrolysis. It is not harmful to polyunsaturated fatty acids, and produces no methyl esters as secondary products. ■

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