papers and notes on methodology

Phospholipid molecular species quantitation from mass spectra of underivatized lipids[1]

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Abstract Isobutane chemical ionization of 0.05 to 5 μg of phospholipid volatilized from a direct exposure probe provides, in less than a minute, mass spectra from which the component molecular species can be quantitated. Molecular response factors are determined from a stable external standard mixture containing saturated molecular species; a simple series of linear equations is used to correct for isotope effect and hydrogen abstraction. An internal standard allows absolute quantitation of the sample. In cases where different molecular species have the same molecular weight, the species can be quantified by tandem mass spectrometry daughter ion analysis.—**Crawford, C. G., and R. D. Plattner.** Phospholipid molecular species quantitation from mass spectra of underivatized lipids. *J. Lipid Res.* 1984. **25:** 518–522.

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The determination of phospholipid molecular species is an important aspect of lipid biochemistry, and recently mass spectrometric analysis of membrane lipids has provided molecular detail that was previously unobtainable. The ability to analyze molecular species by mass spectrometry has, for the most part, been due to the use of stable tertiary-butyldimethylsilyl ether derivatives of diacyl glycerols formed following phospholipase C hydrolysis of the original phospholipid (1). When coupled gas–liquid chromatography–mass spectrometry analysis is carried out on these derivatives, the molecular species composition of rat (2) and human (3) plasma lipoproteins, microsomal (4) and ciliary (5) lipids from *Tetrahymena*, and brain phospholipids (6) have been revealed in exquisite detail.

The development of "soft" ionization modes and improvements in instrumentation (7) have extended mass spectrometric analysis to labile and nonvolatile compounds, including phospholipids. Intact phospholipids have been analyzed by field desorption (8), chemical ionization (CI) (9), and fast atom bombardment (10), which has led to the determination of the molecular species composition of underivatized phospholipids (11, 12). Our previous results (13) from chemical ionization mass spectrometry analysis of intact phosphatidylcholine (PC) indicated the possibility of quantitating molecular species of intact phospholipids. Here we report the results of studies on quantitative analysis of phospholipid molecular species.

MATERIALS AND METHODS

Lyso phosphatidylcholine (PC), purchased from Lipoid KG, Papenburg, West Germany, was used to prepare palmitoyl–linoleoyl (16:0–18:2), palmitoyl–linolenoyl (16:0–18:3), stearoyl–linoleoyl (18:0–18:2), oleoyl–linoleoyl (18:1–18:2), and stearoyl–linolenoyl (18:0–18:3) PC by the method described by Mason, Broccoli, and Huang (14); dilinoleoyl (di-18:2) PC was prepared by the method of Warner and Benson (15). Soy and egg PC were purchased from Sigma Co., St. Louis, MO, and all other phospholipids were purchased from Avanti Polar Lipids, Birmingham, AL.

Transmethylation and gas–liquid chromatographic analyses were carried out as described (16). All mass spec-

Abbreviations: PC, phosphatidylcholine; CI, chemical ionization; GLC, gas–liquid chromatography.

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tra were obtained on a Finnigan 4535/TSQ tandem quadrupole instrument. Conventional mass spectra were obtained by passing all ions through quadrupoles 1 and 2 and scanning with quadrupole 3. Daughter ion spectra were obtained by setting the first quadrupole to allow only those ions of interest to pass into the collision chamber, then scanning with the third quadrupole for collision-activated dissociation products.

Samples were introduced into the mass spectrometer by the direct exposure chemical ionization technique (17). Routinely, 1 μl of sample (0.05–5 μg) was applied to the probe tip in $CHCl_3$ and allowed to dry; then the probe was inserted into the ion source. The filament was heated by programming the current from 0–400 ma at 20 ma/sec. Phospholipids began to vaporize from the probe at 200 ma (ca 200°C) and were completely vaporized in 5–6 sec. Data were collected throughout the total run of 30 sec by utilizing either a multiple ion detecting mode or by scanning over the mass range of m/z 500–800 and then summing across the phospholipid peak.

RESULTS

The major ion formed from phospholipids under chemical ionization conditions contains both fatty acyl groups (9, 13); therefore, this ion was selected to quantitate molecular species. Samples were prepared that contained 1 μg/μl dipentadecanoyl (di-15:0) PC and 5–95 μg/μl of other disaturated PC molecular species. An aliquot of each sample was transmethylated and analyzed by gas–liquid chromatography to determine the amount of each molecular species relative to di-15:0 PC.

Fig. 1 shows the results of a series of experiments where each sample was analyzed under ammonia CI conditions, monitoring the diglyceride ion, $[MH-183]^+$, for each molecular species in the mixture. Amounts were calculated by comparing the area of the di-15:0 PC diglyceride ion (m/z 523) to the area of the diglyceride ion of the other species. The amount calculated from the diglyceride ion area for each species was linear across the range of 50–1000 ng, but there was a decrease in response with increasing molecular weight. These results indicated that quantitation of molecular species was possible if reliable response factors could be determined.

Ammonia chemical ionization of PC produces ions at MH^+, $[MH-42]^+$, and $[MH-183]^+$. Due to the natural abundance of ^{13}C isotope, significant peaks will be found at 1 and 2 amu above each ion; hydrogen abstraction produces smaller peaks at 1 and 2 amu below each ion. A computer program was written that calculated the m/z value for the MH^+, $[MH-42]^+$, and $[MH-183]^+$ ions for any molecular species composition and identified any of the three ions ± 2 amu of a molecular species that would interfere with ions from any other molecular species. We found, for example, that the $[MH-42]^+$ peak (m/z 664) in di-15:0 PC is 1 amu from the $[MH-183]^+$ peak (m/z 663) in di-20:0 PC, which would complicate the quantitation of that molecular species.

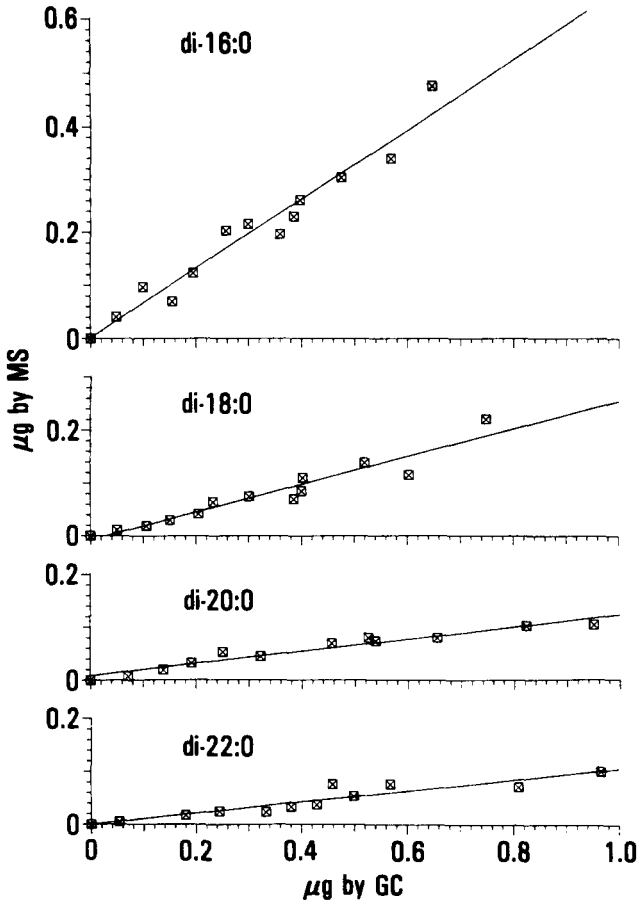

Fig. 1. Comparison of the amount of individual molecular species, relative to di 15:0 PC, when determined by mass spectrometry or gas–liquid chromatography.

When isobutane is used as the reagent gas, the MH^+ ions are less intense than those seen in the corresponding ammonia chemical ionization spectra, but there are no ions between the MH^+ ion and the $[MH-183]^+$ ion that would significantly interfere with the quantitation of any other molecular species.

A standard was prepared of equal weight amounts of the saturated species di-15:0 to di-22:0 PC and analyzed using isobutane as the chemical ionizing reagent gas. The peak heights of the $[MH-183]^+$ ion for each molecular species appeared to decrease in an exponential manner. **Fig. 2** shows that when the area of the $[MH-183]^+$ (m/z 523) ion of di-15:0 PC is defined as 10, the area of the $[MH-183]^+$ ions for each molecular species decreased as a logarithmic function, from which response factors for any amu can be calculated.

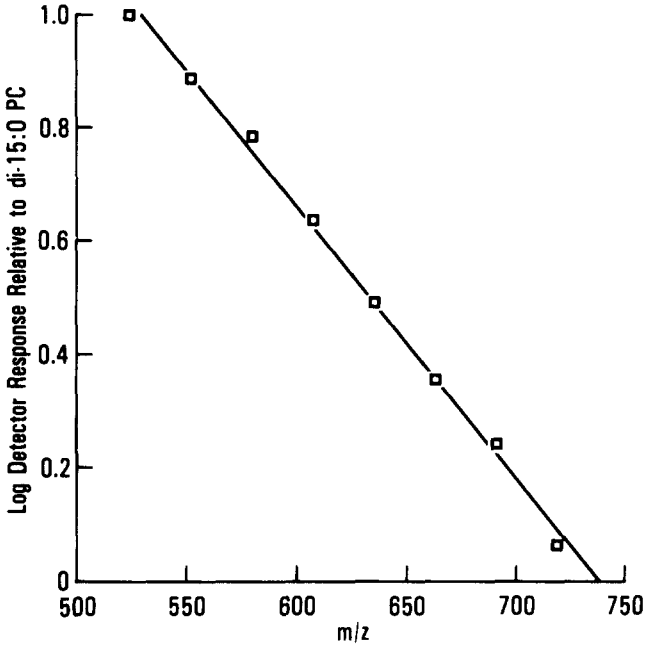

Fig. 2. Detector response to MH^+-183 ions in standard containing equal amounts of di 15:0, 16:0, 17:0, 18:0, 19:0, 20:0, 21:0, and 22:0 PC.

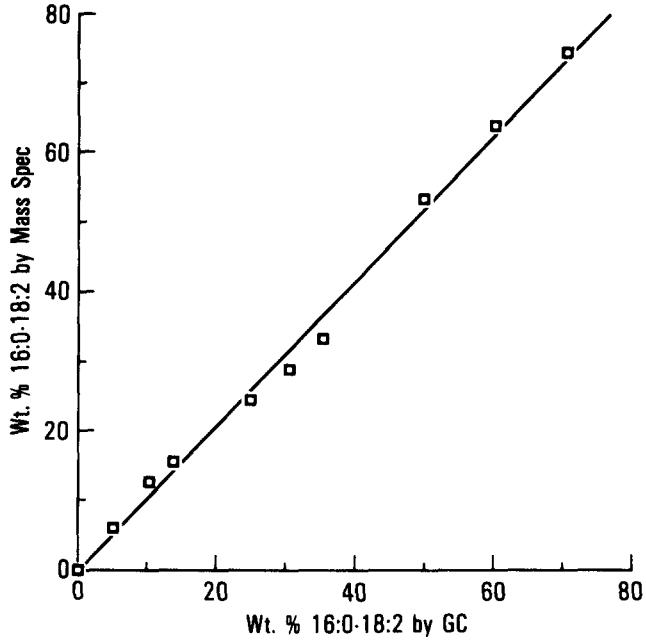

Fig. 3. Comparison of the weight percent of 16:0–18:2 in mixtures of 16:0–18:1, 16:0–18:2, and 16:0–18:3 as determined by gas–liquid chromatography and mass spectra after correction for ion overlap.

We next addressed the problem of quantitating molecular species that differ by only 2 amu. For example, in the spectrum of 16:0–18:2 PC, the diglyceride ion occurs at m/z 575 with peaks at m/z 576 and 577 due to natural isotopic abundances. The peak at m/z 577 in 16:0–18:2 PC would interfere with quantitation of 16:0–18:1 PC, which has the $[MH\text{-}183]^+$ ion at m/z 577. Also seen are peaks at m/z 574 and 573, which arise from hydrogen abstraction; the peak at m/z 573 would interfere with the quantitation of 16:0–18:3 PC.

Spectra of pure 16:0–18:0, 16:0–18:1, 16:0–18:2, and 16:0–18:3 PC provided coefficients for a system of linear equations with a general form of $0.001\ (m/z - 4) + 0.055\ (m/z - 2) + 1\ (m/z) + 0.1\ (m/z + 2) + 0.1\ (m/z + 4)$ used in the matrix solution. Samples were prepared containing 1 $\mu g/\mu l$ of di-15:0 PC and a total of 1 $\mu g/\mu l$ of a mixture of the four species 16:0–18:0, 16:0–18:1, 16:0–18:2, and 16:0–18:3 PC; the samples then were analyzed by gas–liquid chromatography and mass spectrometry. Corrected areas were calculated for the ions at m/z 573, 575, 577, and 579 by the above coefficients, and the weight percentage of the 16:0–18:2 species is shown in **Fig. 3.** As can be seen, there is excellent correlation between the GLC results and the amount of 16:0–18:2 determined by mass spectrometry after making corrections for the interference by other ions. Quantitation of the other molecular species in the sample (data not shown) exhibited the same correlation between mass spectrometric and GLC analysis.

In most samples of phospholipids from biological sources there are different molecular species that have identical molecular weights and, therefore, cannot be resolved by a single mass spectrometer; however, quantitation can be achieved utilizing the MS/MS capabilities of the instrument used for this study. Mixtures of molecular species with identical molecular weight were prepared, i.e., 18:0–18:2 and di 18:1 PC, 18:1–18:2 and

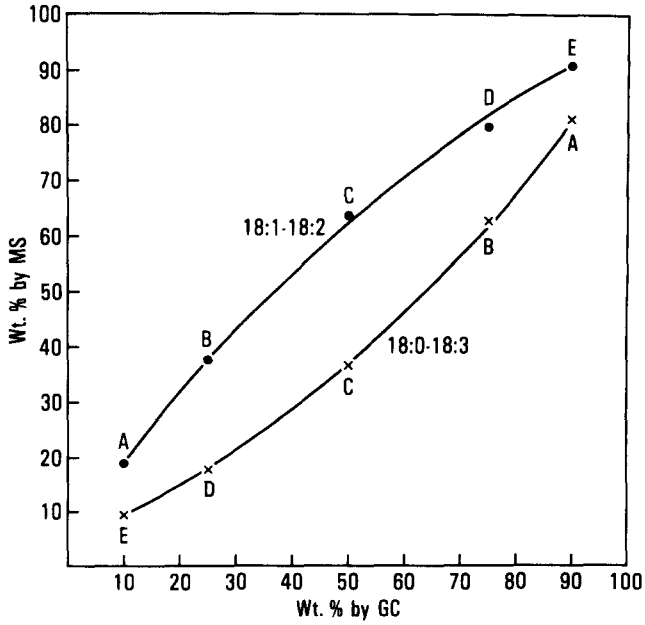

Fig. 4. Comparison of amounts of 18:1–18:2 and 18:0–18:3 PC as determined by gas–liquid chromatography and daughter ion spectra. The samples were prepared with 18:1–18:2/18:0–18:3 PC ratios of: A (1:9), B (1:3), C (1:1), D (3:1), and E (9:1).

TABLE 1. Weight percent of molecular species as determined by mass spectrometry

m/z	Molecular Species	Soy PC		Soy PE	Egg PC	
551	16:0–16:0				0.8	
549	16:0–16:1				1.0	
579	16:0–18:0				0.5	
577	16:0–18:1	2.7		5.6	39.8	
575	16:0–18:2	25.7		44.0	21.9	
573	16:0–18:3	2.5		2.5	0.8	
605	18:0–18:1	0.9		0.7	10.1	
603	18:0–18:2	9.5	8.5[a]	7.0	13.2	10.6[a]
	18:1–18:1		1.0[a]			2.6[a]
601	18:1–18:2	12.5	11.9[a]	11.3	2.8	
	18:0–18:3		0.6[a]			
599	18:2–18:2	38.9		24.1		
	16:0–20:4				2.5	
597	18:2–18:3	6.9		3.3		
595	18:3–18:3	0.4		1.4		
627	18:0–20:4				3.9	
623	16:0–22:6				1.9	
651	18:0–22:6				0.6	

[a] Determined from daughter ion spectra.

18:0–18:3 PC, and 16:0–20:4 with di 18:2 PC. When the $[MH-183]^+$ is selected as the parent ion, passed into the collision chamber, and scanned with quadrupole 3, the RCO^+ ion for each fatty acid of the individual molecular species is observed, and the amount of each molecular species can be calculated by adding the areas for the fatty acids in the molecular species. **Fig. 4** shows the data on quantitation from daughter ion spectra of mixtures containing 18:1–18:2 and 18:0–18:3 PC. Even though the amounts determined by daughter ion experiments were not directly proportional to those found by GLC analysis, a usable calibration curve was obtained.

Table 1 shows the results obtained by this technique on the molecular species composition of soy PC, soy phosphatidylethanolamine, and egg PC. The fatty acid composition calculated from the molecular species composition was in good agreement with that found by GLC analysis. For example, the fatty acid composition of soy PC determined by GLC or calculated from the species data was respectively: 16:0 (14.3%, 15.4%), 18:0 (3.5%, 5.0%), 18:1 (9.4%, 8.8%), 18:2 (65.5%, 65.4%), 18:3 (7.1%, 5.5%).

DISCUSSION

In our initial attempts to quantitate phospholipid molecular species we used ammonia as the chemical ionization reagent gas, but we found that in our standard mixture the $[MH-42]^+$ ions in di-15:0 PC and di-17:0 PC were 1 amu from the $[MH-183]^+$ peaks in di-20:0 PC and di-22:0 PC, respectively. Although computer analysis of ammonia CI spectra of actual and hypothetical molecular species compositions revealed very few ions in any species that interfered with the ions in other species, the decrease in detector response seen in Fig. 1 indicated that a standard which extended over the range of possible molecular species was desirable. Furthermore, when ammonia was the reagent gas, we found that the ion source in the mass spectrometer was rapidly contaminated and that small changes in reagent gas pressure resulted in large changes in ion intensities. In isobutane CI spectra of phospholipids there are less intense MH^+ ions when compared to ammonia CI spectra, but there are no ions in any molecular species that significantly interfere with the diglyceride ion of any other species. Also, the source was not contaminated as readily and ion intensities were not as sensitive to reagent gas pressure. With isobutane as the reagent gas, we were able consistently to achieve reproducibility to within 10%.

The standard mixture we selected extends across the range of most molecular species. It provided factors to correct for detector response in the form of $\text{area}_{(m/z)}/Ae^{-b(m/z)}$, where A and b are determined from the exponential regression analysis of the results from standard samples.

When molecular species differ by 2 amu, corrections of the ion intensity data are necessary because ions formed by hydrogen abstraction or due to natural isotopic abundance occur that have m/z values identical to the m/z of the diglyceride ion of other species. These corrections can be made by solving a series of linear equations. We determined the coefficients of the equations from spectra of pure 16:0–18:0, 16:0–18:1, 16:0–18:2, and 16:0–18:3 PC, but they can also be calculated from spectra of the standard mixture. In contrast to previous studies (2, 4), we found, at least through the linolenic species, no decrease in intensity of the $[MH-183]^+$ ion as desaturation increased.

When different molecular species have the same molecular weight, the identity of the species cannot be determined from a single mass spectrum; however, MS/MS analysis can identify the species from the RCO^+ daughter ions of the parent diglyceride ion. An estimation of the relative amounts can be calculated from the daughter ion spectra. But, in the mixtures we analyzed, the amount calculated for the species with the most unsaturated fatty acid was lower than the actual amount.

In our previous studies (13), where samples were volatilized from a probe that was neither in the ion beam nor programmable by temperature, we found considerable differences between individual spectra across the phospholipid peak. In the present report, the samples were introduced on the direct exposure probe, which was heated reproducibly at 20°C/sec. We found some differences between the spectra but could not identify a pattern in the differences by selected ion retrieval, either of ions from saturated or unsaturated species or of ions from low- or high-molecular-weight species.

Although we have presented the data in Table 1 as

weight percent, this was determined from the weight of each species calculated from the amount of di-15:0 PC added as the internal standard. Other samples we have analyzed contain 14:0–16:0 PC, which precludes the use of di-15:0 PC; but in those cases, the relative amounts of each species can be calculated.

Elegant methods have been devised to analyze glycerophospholipids by gas–liquid chromatography–mass spectrometry (1, 18), but these techniques require both enzymatic and chemical derivatization of each sample where sample modification and sample loss can occur. On the other hand, they allow the analysis of samples containing both acyl and ether lipids that have an extremely complex molecular species composition, which would be difficult to analyze by the method we are reporting. Recently, Myher and Kuksis (19) have shown that molecular species composition can be determined by GLC analysis of tertiary-butyldimethylsilyl ether derivatives on polar capillary columns, obviating the expense and complexity of the mass spectrometer. Although the phospholipids must be hydrolyzed and derivatized, this method of molecular species determination will have wide appeal because of the comparative simplicity of the instrumentation.

For samples with comparatively simple molecular species composition, we have found that isobutane chemical ionization mass spectrometry used in conjunction with a temperature-programmable direct exposure probe provides a rapid technique to quantitate molecular species of diacyl phospholipids from biological samples. The use of a stable external standard mixture provides a means to monitor the operation of the mass spectrometer, determine the quality of the data being obtained during the course of analysis, and give a response curve for quantitation. The corrections necessitated by loss of detector response, natural isotopic abundance, and hydrogen abstraction are straightforward and verifiable. Preliminary results indicate that the molecular species of other phospholipids, e.g., phosphatidylinositol and phosphatidylglycerol, can also be quantitated by this method.

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