Compositional and molecular species analysis of phospholipids by high performance liquid chromatography coupled with chemical ionization mass spectrometry

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Abstract High performance liquid chromatography (HPLC) was combined with chemical ionization mass spectrometry (CIMS) by the use of a moving-belt interface. The technique was employed for the analysis of naturally occurring phospholipids. Positive and negative ion mass spectra of various phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, and sphingomyelin were obtained in the chemical ionization mode with ammonia or methane as the reagent gas. Specific ions for individual phospholipid "bases" were identified. These ions were used in specific ion monitoring of the phospholipids during HPLC–CIMS. CIMS of each phospholipid also provided extensive information on the molecular species of the individual class of phospholipids. Relative abundance of different molecular species of each phospholipid as determined by CIMS agreed well with the results obtained by gas–liquid chromatography. Rat brain phospholipids were analyzed by HPLC–CIMS in about 15 minutes. Routinely, about 5 μg of individual phospholipid was analyzed by HPLC–CIMS, however with specific ion monitoring the method provides a detection capability at the subnanogram level.—**Jungalwala, F. B., J. E. Evans, and R. H. McCluer.** Compositional and molecular species analysis of phospholipids by high performance liquid chromatography coupled with chemical ionization mass spectrometry. *J. Lipid Res.* 1984. **25:** 738–749.

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Phospholipids are important constituents of all cell membranes. Various methods have been published for the analysis of these lipids from biological sources. However, very few reports have been published on the analysis of various phospholipids by HPLC (reviewed in 1, 2) and, to our knowledge, combined use of HPLC–CIMS for the analysis of phospholipids has not been previously reported. The most convenient detection method for the HPLC analysis of these lipids is by monitoring the elution with an ultraviolet detector in the 200 nm range (3, 4). However, this method of detection does not allow direct quantitation of the separated phospholipids since the response varies with the number of functional groups, primarily the number of double bonds in the phospholipid molecules (3). Again, severe limitations are imposed in the choice of chromatographic solvents used since solvents that absorb ultraviolet light at 200 nm cannot be used. HPLC–MS of phospholipids can overcome these limitations besides providing extensive information on the structural identification of each phospholipid with sensitivity previously not possible (<1 μg range).

Recently we reported a HPLC–MS method for the analysis of sphingoid bases from sphingolipids with the use of a moving-belt transport type interface for coupling of HPLC to mass spectrometry (5). Here we describe the use of such an interface for the HPLC–MS analysis of phospholipids. Chemical ionization mass spectrometry of various intact phospholipids is presented. It is shown how this information can be directly employed to determine the relative abundance of each molecular species of individual class of phospholipids within a short time interval with high sensitivity. Part of this work has previously appeared in abstract form (6).

MATERIALS AND METHODS

Synthetic and naturally occurring phospholipid standards were purchased from Supelco (Bellefonte, PA) and

Abbreviations: HPLC, high performance liquid chromatography; MS, mass spectrometry; CI, chemical ionization; GLC, gas–liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SP, sphingomyelin; GPE, glycerylphosphoethanolamine; PG, phosphatidylglycerol.

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used without further purification. Phospholipids from rat brain and other tissues were extracted and purified as previously described (7).

HPLC was performed with a Spectra Physics (model 8700) gradient pump and a Brownlee cartridge, 6 cm × 2.0 mm spherical silica (5 μm) column. The column was eluted with a linear gradient of solvent A, dichloromethane–methanol–water 93:6.5:0.5 (by volume) and solvent B, dichloromethane–methanol–water–15 M NH_4OH 65:31:4:0.2 (by volume), from 12% B to 45% B in 10 min and programming to 100% B in 2 min, at a flow rate of 0.8 ml/min. The sample to be injected was dissolved in dichloromethane–methanol–15 M ammonia 1:1:0.01 (by volume) at a concentration of about 1 μg phospholipid per 1 μl. The HPLC solvent was applied as a fine stream to the moving belt of a Finnigan HPLC–MS interface to achieve an even coating. The stream was formed by inserting a 10-cm section of polyimide-coated fused silica capillary GLC column (0.25 mm i.d.), pulled to a fine orifice, into the bore of the 0.009 in i.d. × $^1/_{16}$ in Teflon tube from the HPLC column. A $^1/_{16}$ in Swagelok fitting was then tightened over the sleeved portion of the tubing to provide a compression seal. The sample evaporator heater was at 330°C and the clean-up heater was at 210°C. A Finnigan model 4500 mass spectrometer was used with ammonia (0.75 torr) or methane (0.6 torr) as the CI reagent gas. The ion source was at 150°C. Positive and/or negative ion spectra were obtained by the system under the control of a Teknivent model 56K MS data system. MS data were collected from m/z 100–900 with 5–7-second scans. Gas–liquid chromatography of the fatty acid esters was as previously described (8).

RESULTS

CIMS of phospholipids with the use of moving-belt interface

Positive and negative chemical ionization mass spectra of various synthetic and naturally occurring phospholipids were obtained with ammonia and methane as the reagent gases. The phospholipid, about 5 μg in 10 μl of solvent, was directly applied to the moving belt with a microsyringe. Mass spectra of various synthetic and naturally occurring phospholipids were studied.

Phosphatidylcholine. CIMS of synthetic 18:0–18:1 PC with ammonia and methane as reagent gases in the positive ion mode is shown in Fig. 1A and B, respectively, as an example. The major characteristic ions and ions in high mass range of each phospholipid are listed in **Table 1.** The following ions were recognized in positive ion-ammonia CIMS of PC: $[M + 1]^+$, $M^+ - 41$ ($[M + NH_4]^+ - N(CH_3)_3$), $M^+ - 182$ (i.e., M^+ − phosphocholine) and $M^+ - 147$ ($[M + N_2H_7]^+ - 182$, diglyceride adduct). The positive ion-ammonia CIMS of lung PC is shown in Fig. 1C, and the ions that provide fatty acid species information are listed in **Table 2.** Nineteen different molecular species of lung PC were identified from this spectrum.

From the relative intensity of these diglyceride-related ions one can directly calculate the percentage composition of the molecular species present in each phospholipid. Calculation of the relative composition of major species of lung PC from the relative intensity of three different ions in the mass spectrum gave results that were very similar (Table 2). Good correspondence of results was obtained with $M^+ - 41$ and $M^+ - 182$ ions, because they are major ions in the spectrum. The results from $[M + 1]^+$ (not shown) or $M^+ - 147$ ions showed some differences due to their relatively low intensity. The total fatty acid species of the lung PC was also analyzed by GLC and the relative percent distribution is given in **Table 3.** The total fatty acid composition of lung PC was calculated from MS data (Table 2) by adding the relative amounts of each fatty acid in various molecular species and then dividing by two. The percent distribution of fatty acids as analyzed by GLC was very similar to that as calculated from the MS data (Table 3). This result indicated that the molecular fatty acid species assignments as done by MS are mostly correct. Small overlap cannot be ruled out since the molecular weights of different combinations of fatty acids can be the same; for example, the molecular weights of 16:0–20:4, 18:2–18:2, and 18:1–18:3 PC are the same. In nature, with some exceptions, both fatty acids of phospholipids are not unsaturated, therefore, generally, the assignments based upon one saturated and one unsaturated fatty acid were made. Analysis of monoglyceride ions and free fatty acid ions (**Fig. 1**) at the lower end of the MS (m/z 250–500) also helped to eliminate certain possibilities. Reversed phase HPLC (9, 10) with on-line MS would distinguish the various fatty acids-containing PC with the same molecular weight.

Besides the above-mentioned diglyceride-related ions in the positive ion CIMS, the phospholipid "base"-related ions were identified (Table 1). In the case of PC and also SP, m/z 142 (100%, not shown in Fig. 1A, C), 156, 172, 184, 186, and 196 were identified. M/z 142 was the most characteristic ion for choline-containing phospholipids and was used, as shown later, for monitoring these phospholipids after HPLC.

In the ammonia and methane CIMS of synthetic and natural PC, the fatty acid and monoglyceride-related ions were identified between m/z 200–500 and are shown in Fig. 1. For example, in the ammonia CIMS of 18:0–18:1-containing PC (Fig. 1A) the 18:0 (*sn*-1 position)-containing ions are at m/z 267, 284, 301, 341, 358, 376, 435, 452, and 492, whereas the corresponding 18:1 (*sn*-2 position)-

TABLE 1. Major characteristic fragment ions in the positive and negative ammonia and methane CIMS of various phospholipids

	Ammonia CI		Methane CI	
Phospholipid	Positive	Negative	Positive	Negative
PC	142 (from phosphocholine) $[M + 1]^+$ $M^+ - 41$ $M^+ - 182$ $[M + 35]^+ - 182$	182, 123 $[M - 1]^-$ $M^- - 13$ $M^- - 24$ $M^- - 33$ $M^- - 42$ $M^- - 60$ $M^- - 184$	125, 139, 153 $[M + 1]^+$ $M^+ - 41$ $M^+ - 182$	159, 182 $[M - 1]^-$ $M^- - 13$ $M^- - 24$ $M^- - 33$ $M^- - 42$ $M^- - 60$ $M^- - 130$ $M^- - 148$ $M^- - 184$
PE	141 (ethanolamine phosphate) $M^+ - 140$ $[M + 35]^+ - 140$ $[M + 17]^+ - 140$		124 $M^+ - 140$	191 $M^- - 88$
PS	105 (serine) $M^+ - 184$ $[M + 35]^+ - 184$		 $M^+ - 184$	139, 173 $M^- - 132$
PI	180 (inositol) 198 (inositol +18) $M^+ - 259$ $[M + 35]^+ - 259$		 $M^+ - 259$	 152, 179 $M^- - 207$
SP	142 (from phosphocholine) $M^+ - 182$ $M^+ - 182 - 18$		 $M^+ - 182$ $M^+ - 182 - 18$	
Lyso PE	141 (ethanolamine phosphate) $M^+ - 140$ $[M + 35]^+ - 140$ $M^+ - 43$ $M^+ - 17$			
Lyso PC	142 (from phosphocholine) $M^+ - 182$ $[M + 35]^+ - 182$ $M^+ - 31$ $M^+ - 41$			
PG	172 (glycerol phosphate) $M^+ - 171$ $[M + 35]^+ - 171$			

containing ions are at 2 less mass units and are clearly identified. Even in a complex mixture of naturally occurring phospholipid such as lung PC, the fatty acid-related ions can be identified (Fig. 1C); for example, for dipalmitoyl-containing PC, the ions related to 16:0 are as expected, at m/z 239, 256, 273, 313, 330, 348, 407, 424, and 464. In the methane CIMS of 18:0–18:1-containing PC (Fig. 1B), the fatty acid ions, except the methane adduct ions, are at the same position as in the ammonia CIMS. The methane adduct ions are at expected mass units which correspond to the ammonia adduct ions in the former spectrum.

The negative ion-ammonia CIMS of PC differed considerably from the positive ion spectra. They contained $[M - 1]^-$, $M^- - 13$, $M^- - 33$, $M^- - 42$, $M^- - 60$, and $M^- - 184$ as the major ions in the high mass range. The choline base was represented by m/z 182 and 123. M/z 123 presumably results from m/z 182 by loss of trimethylamine.

The positive ion-methane CIMS of PC was very similar to the ammonia CIMS in the mass range 500–800, which gives characteristic information of the diglyceride moiety of the molecule. However, in the lower mass range, the strongest ions were m/z 125, 139, and 153 related to

TABLE 2. Molecular species of lung phosphatidylcholine by ammonia chemical ionization mass spectrometry

Molecular Species	$M^+ + 1$	$M^+ - 41$	$M^+ - 147$	$M^+ - 182$
14:0–16:0	706	664 (9.1)	558 (6.2)	523 (7.6)
15:0–16:0	720	678 (2.0)	572 (2.3)	537 (1.9)
14:0–18:3	728	686 (0.5)	580 (1.1)	545 (1.1)
14:0–18:2	730	688 (2.1)	582 (2.3)	547 (2.2)
16:0–16:1	732	690 (12.8)	584 (9.9)	549 (11.4)
16:0–16:0	734	692 (29.2)	586 (23.0)	551 (28.3)
17:0–18:1	746	704 (0.7)	598 (1.5)	563 (0.9)
16:0–18:3	756	714 (1.4)	608 (3.0)	573 (1.5)
16:0–18:2	758	716 (11.0)	610 (9.9)	575 (10.6)
16:0–18:1	760	718 (13.9)	612 (12.1)	577 (12.8)
16:0–18:0	762	720 (3.6)	614 (5.3)	579 (4.5)
16:0–20:4		740 (3.4)	634 (4.5)	599 (4.5)
18:0–18:3		742 (2.3)	636 (4.2)	601 (2.8)
18:0–18:2	786	744 (3.4)	638 (5.3)	603 (3.9)
18:0–18:1	788	746 (1.6)	640 (2.6)	605 (1.9)
16:0–22:6		764 (0.6)	658 (1.5)	623 (0.9)
16:0–22:5		766 (0.6)	660 (1.5)	625 (0.9)
18:0–20:4		768 (1.8)	662 (3.8)	627 (2.2)
16:0–22:3		770 (0.1)	664 (—)	629 (0.1)

Numbers in parentheses represent the mole percent composition of the fatty acid species of the phospholipid as calculated from the relative intensity of the indicated ion. In the case of $M^+ + 1$ these values are not reported inasmuch as some of the species did not present the expected $M^+ + 1$ ions. The values are the average of three determinations.

the choline base. The negative ion methane CIMS of PC contained $M^- - 1$, $M^- - 13$, $M^- - 24$, $M^- - 33$, $M^- - 42$, $M^- - 60$, $M^- - 130$, $M^- - 148$, and $M^- - 184$ in the high mass range. The characteristic choline-derived ions were m/z 159 and 182.

Phosphatidylethanolamine. In the positive ion-ammonia CIMS of phosphatidylethanolamine, $M^+ - 140$ (M^+ − phosphoethanolamine) and $[M + 35]^+ - 140$ were the major ions in the high mass range. $[M + 17]^+ - 140$ was also present with low intensity. The characteristic ion for ethanolamine base was at m/z 141, with highest intensity in the spectrum. This was useful in monitoring this phospholipid after HPLC. The positive ion-ammonia CIMS of bovine brain ethanolamine glycerophospholipids is shown in **Fig. 2.** Bovine brain PE contains both diacyl and alkylacyl glycerylphosphoethanolamine (GPE), the majority being alk-1-enyl, acyl glycerylphosphoethanolamine. The $M^+ - 140$ and $M^+ - 105$ ions for the alk-1-enyl, acyl GPE were distinguished from the corresponding ions derived from diacyl GPE, the former having 14 units less mass than the latter type. They cannot, however, be distinguished from the corresponding odd chain fatty acid-containing diacyl GPE. Based on the intensity of the $M^+ - 140$ ions, the composition of alkenylacyl and alkylacyl GPE was calculated to be 69%, whereas diacyl GPE was 31% of the total PE. This agreed with the values published in the literature for bovine brain PE (11). The major diacyl GPE molecular species tentatively identified were: 18:0–18:1 (26.7%, m/z 605 and 640); 18:0–18:2 (21.7%, m/z 603 and 638); 16:0–18:1 (17.1%, m/z 577 and 612); 18:0–20:4 (15.1%, m/z 627 and 662); 18:0–22:6 (7.1%, m/z 651 and 686); and 18:0–22:5 (5.3%, m/z 653 and 688). The major alkenylacyl and alkylacyl GPE molecular species tentatively identified were: 18:1–18:2 (28.2%, m/z 587 and 622); 16:1–18:1 (24.8%, m/z 561 and 596); 18:1–18:1 (16.8%, m/z 589 and 624); 18:1–20:4 (8%, m/z 611 and 646); and 18:0–20:4 (4%, m/z 613 and 648). About 15 other molecular species of PE in small quantities were identified but are not listed here.

The positive ion-methane CIMS of PE was similar to that of positive ion-ammonia CIMS in the high mass range in having $M^+ - 140$ as the major ion, whereas $M^+ - 105$ and $M^+ - 123$ were only of lower intensity. However, the characteristic ion for ethanolamine phospholipids was at m/z 124 in these spectra. In negative ion-methane CIMS of PE, the major ion in the high mass range was $M^- - 88$, whereas the characteristic ion for ethanolamine glycerophospholipid was at m/z 191.

Phosphatidylserine. In the positive ion-ammonia CIMS of phosphatidylserine the major ions were $M^+ -$ phosphoserine ($M^+ - 184$) and $[M + 35]^+ - 184$ in the high mass range. The characteristic ion of all serine-containing phospholipids was at m/z 105. The major molecular species of bovine brain PS (**Fig. 3**) were identified by MS as 18:0–18:1 (54%, m/z 605 and 640); 18:0–18:2 (7%, m/z 603 and 638); 18:0–20:1 (17%, m/z 633 and 668); 18:0–20:2 (6.3%, m/z 631 and 666); 18:0–22:2 (2%, m/z 694); and 16:0–18:1 (1%, m/z 577 and 612) with minor amounts of other species. Small amounts of al-

TABLE 3. Percent fatty acid composition of lung phosphatidylcholine by gas–liquid chromatography and mass spectrometry

Fatty Acid	GLC	MS		
		$M^+ - 41$	$M^+ - 147$	$M^+ - 182$
14:0	2.1	5.8	4.8	5.4
15:0	0.8	1.0	1.1	1.0
16:0	53.2	58.1	50.6	56.2
16:1	6.5	6.2	5.0	5.7
17:0	0.5	0.3	0.8	0.5
18:0	8.0	7.0	11.3	8.1
18:1	9.8	8.1	8.1	7.7
18:2	9.9	8.2	8.7	8.4
18:3	2.2	2.1	4.1	2.7
22:5	0.5	0.3	0.7	0.5
20:4	5.0	2.6	4.1	3.3
22:3	0.5	0.05		0.05
22:6	1.0	0.3	0.7	0.5

The values are the average of three separate analyses. The percentage fatty acid composition by MS was calculated from $M^+ - 41$, $M^+ - 147$, and $M^+ - 182$ ions by summation of the percentage of individual fatty acid in different molecular species (Table 2) and dividing by two.

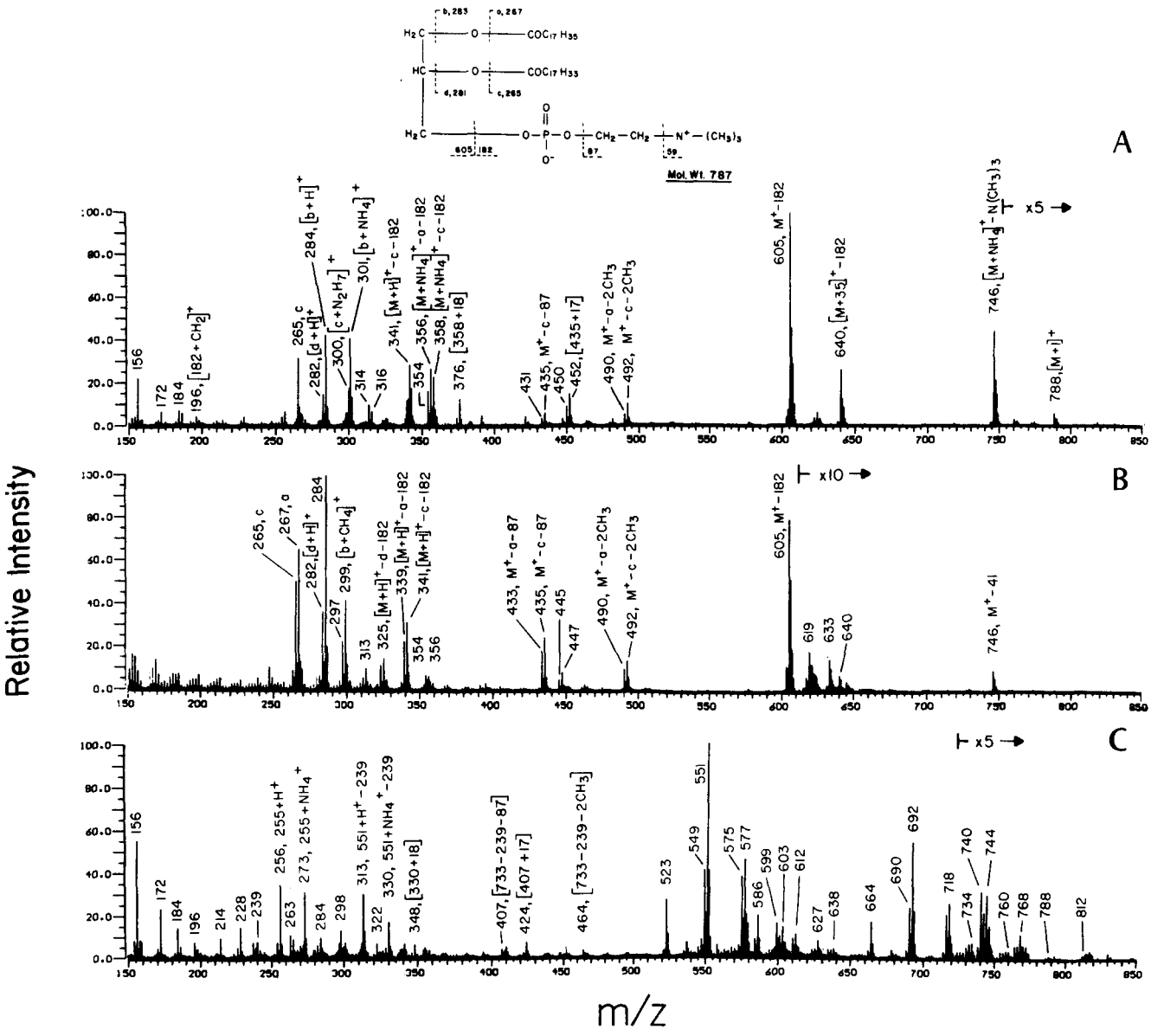


Fig. 1. Ammonia (A and C) and methane (B) chemical ionization mass spectra of synthetic 18:0–18:1 containing PC (A and B) and lung PC (C) in the positive ion mode. About 5 μg of PC was applied to the moving belt of the Finnigan model 4500 mass spectrometer interface. MS data were collected from m/z 150–850 in the chemical ionization mode with ammonia or methane (0.75 torr) as the reagent gas, every 6 seconds. The ion source temperature was 150°C. Fragmentation of 18:0–18:1 containing PC is shown in the figure. Note the ordinate scale expansion for A, fivefold from m/z 755; B, tenfold from m/z 615, and C, fivefold from m/z 725; indicated at the top of each spectrum. $M^+ + 1$, $M^+ - 41$, $M^+ - 147$, and $M^+ - 182$ ions of various molecular species of lung PC (C) are listed in Table 2.

kenylacyl PS were also identified as 18:1–22:6 (7%, m/z 607 and 642) and 18:1–18:0 (2%, m/z 591 and 626). The fatty acid composition of PS as determined by GLC was in agreement with that as analyzed by CIMS. GLC analysis of the same sample of PS showed major fatty acids as 18:0, 40%; 18:1, 30%; and 20:1, 11%. Calculations from the relative intensity of the $[M + 35]^+ - 184$ ion showed 18:0, 43%; 18:1, 28%; and 20:1, 9% as the major fatty acids.

In positive ion-methane CIMS of PS, the major ion was $M^+ -$ phosphoserine ($M^+ - 184$) with high intensity, whereas $[M + 35]^+ - 184$ was present only in minor amounts. In the negative ion-methane CIMS of PS, m/z 139 and 173 were the major ions in the low mass range, whereas $M^- - 132$ was the major ion in the high mass range.

Phosphatidylinositol. Positive ion-ammonia CIMS of phosphatidylinositol had $M^+ - 259$ ($M^+ -$ phosphoino-

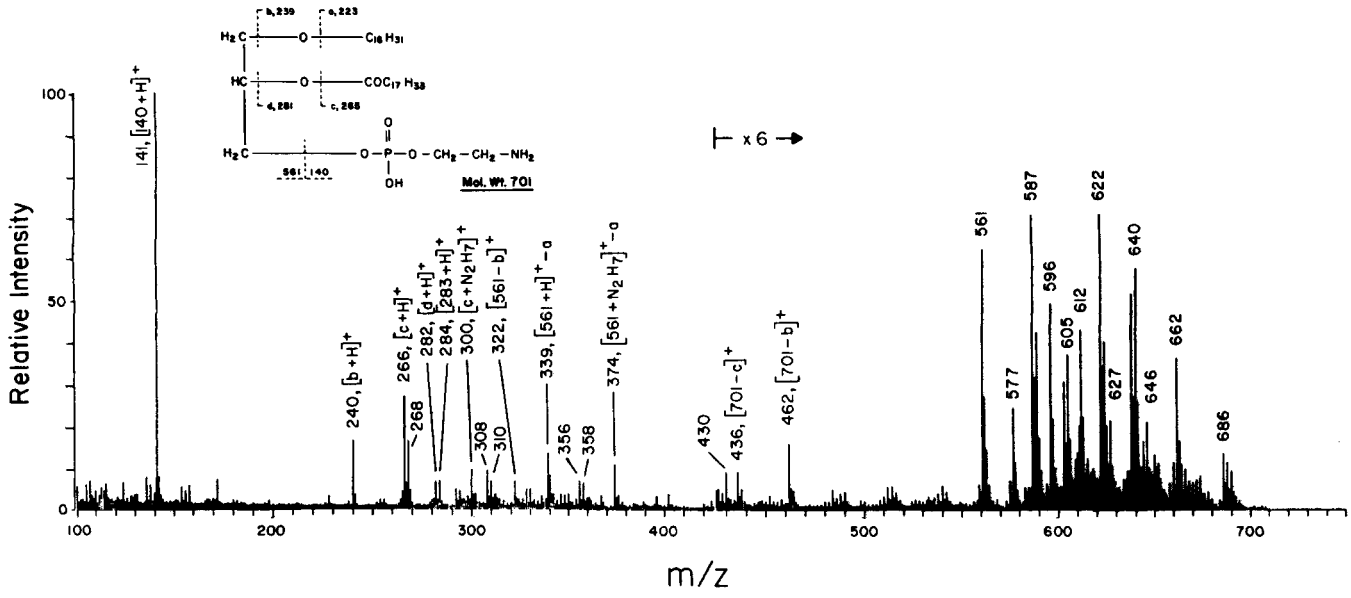


Fig. 2. Ammonia chemical ionization mass spectrum of bovine brain ethanolamine glycerophospholipids (5 μg) in positive ion mode. The spectrum was obtained as described in the legend of Fig. 1. The ordinate scale is expanded sixfold from m/z 425. Fragmentation of 16:1 alkyl–18:1 acyl GPE (plasmalogen) is given as an example.

sitol) and $[M + 35]^+ - 259$ as the major ions in the high mass range. The characteristic ions of PI were m/z 198 and 180. The major molecular species of bovine brain PI (**Fig. 4**) were identified as: 18:0–20:4 (40%, m/z 627 and 662); 18:0–18:2 (10%, m/z 603 and 638); 18:0–18:1 (14%, m/z 605 and 640); 18:0–20:3 (11%, m/z 629 and 664). Nine other molecular species were identified which included, 16:0–18:2 (2%, m/z 575 and 610);

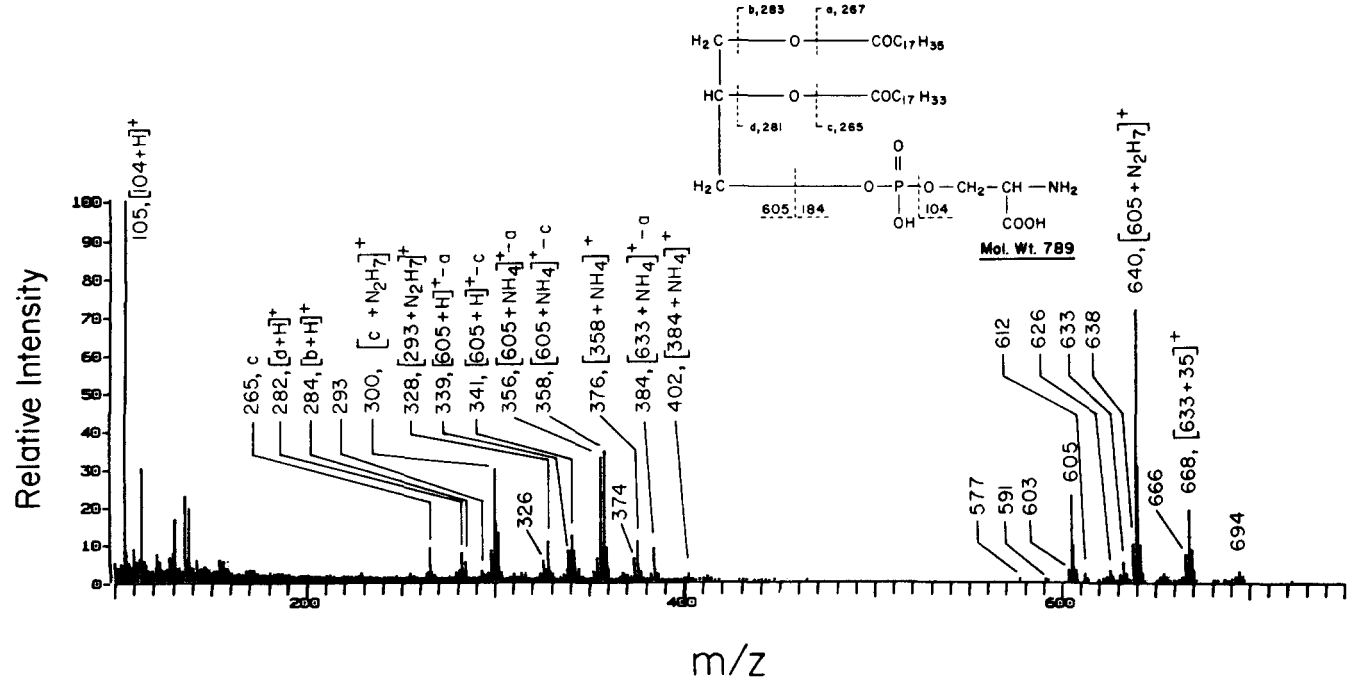


Fig. 3. Ammonia chemical ionization mass spectrum of bovine brain phosphatidylserine (5 μg) in positive ion mode. The spectrum was obtained as described in the legend of Fig. 1. Fragmentation of 18:0–18:1 glycerophosphoserine is given as an example. Ions m/z 293, 328, 384, and 402 are from 18:0–20:1 fatty acid containing PS.

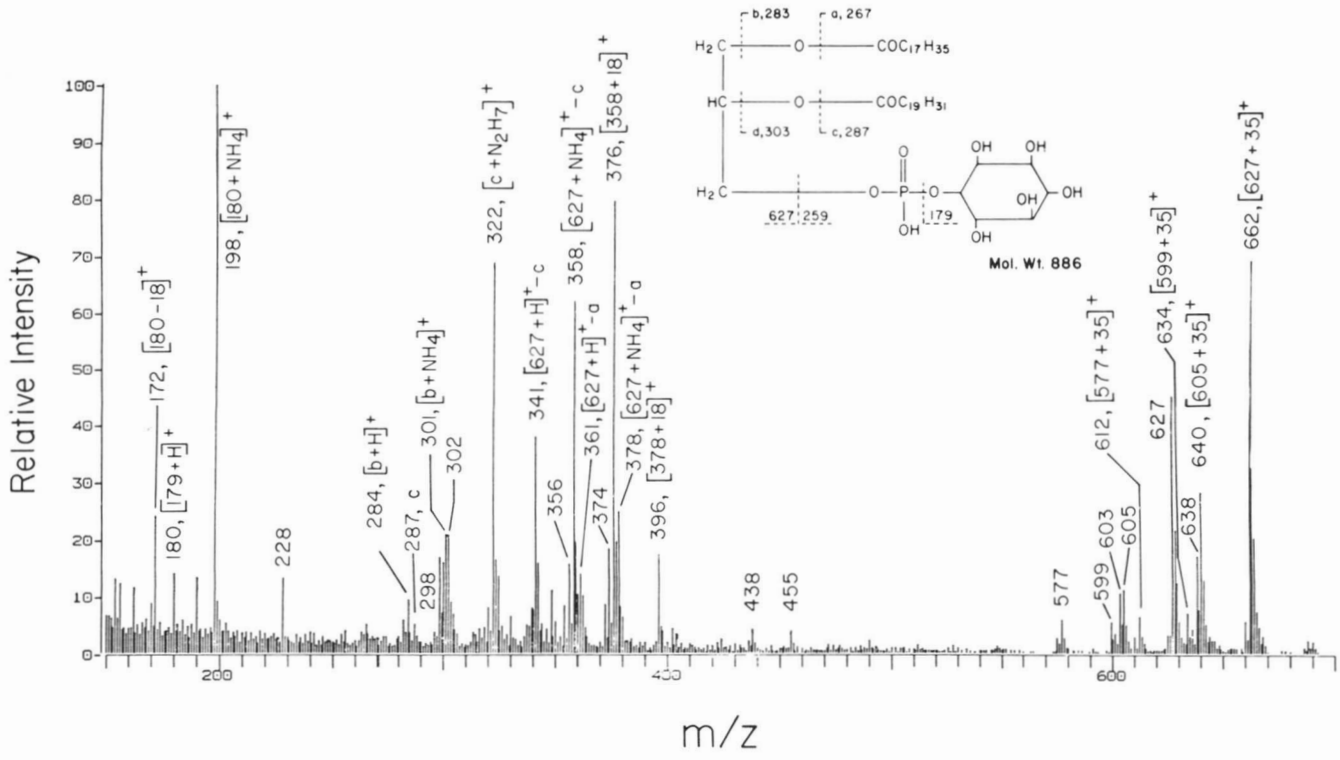


Fig. 4. Ammonia chemical ionization mass spectrum of bovine brain phosphatidylinositol (5 μg) in positive ion mode. The spectrum was obtained as described in the legend of Fig. 1. Fragmentation of 18:0–20:4 glycerophosphoinositol is given as an example.

16:0–18:1 (4%, m/z 577 and 612); 16:0–20:4 (4.5%, m/z 599, 634); and 18:0–20:2 (2.6%, m/z 631 and 666). GLC analysis of fatty acid methyl esters obtained from the same sample agreed well with the calculated amounts of the fatty acids as determined by CIMS.

In the positive ion-methane CIMS of PI, the major ion was at $M^+ - 259$. $[M + 35]^+ - 259$ was present with low intensity. In the negative ion-methane CIMS of PI, $M^- - 207$ was the major ion.

Sphingomyelin. The major high molecular weight ions in the positive ion-ammonia CIMS of sphingomyelin were the ceramide ions $M^+ - 182$ and $[M^+ + 18] - 182$. The ceramide ion is easily distinguished from the diglyceride ions in being of even number (because of the nitrogen) as opposed to the odd-numbered diglyceride ion. The major molecular species of bovine brain sphingomyelin identified by positive ion-ammonia CIMS (**Fig. 5**) were: C_{18} sphingenine with 18:0 (48%, m/z 530 and 548); 16:0 (4%, m/z 502 and 520) 20:0 (5%, m/z 558 and 576); 22:0 (6%, m/z 586 and 604), 24:1 (7%, m/z 612 and 630); and 24:0 (7%, m/z 614 and 632). Small amounts of molecular species with C_{18}-sphinganine and fatty acid 18:0 (10%, m/z 550 and 532), 20:0, 22:0, and 24:0 were also identified. C_{20}-sphingenine with 16:0 fatty acid cannot be resolved from C_{18}-sphinganine with 18:0 fatty acid. However, C_{18}-sphinganine and C_{20}-sphingenine-containing sphingomyelins are about 7% and 10%, respectively, in bovine brain sphingomyelin (12). The GLC analysis of fatty acid agreed with CIMS analysis. The positive ion-methane CIMS of SP was similar to that obtained with ammonia as the reagent gas.

HPLC–MS of phospholipids

HPLC–MS of standard phospholipid mixture was performed with a variety of solvents and columns. The MS monitoring was generally done with ammonia as a reagent gas in the positive ion mode. Initially, silica gel column (Accupak 3 μm, 4.6 mm i.d. × 10 cm) with a gradient solvent mixture of dichloromethane–methanol–water–acetic acid was used. Although excellent separation of all the phospholipid standards was achieved, the separation deteriorated after repeated injections and variable chromatographic resolutions were obtained. The separation of acidic phospholipids PS and PI from each other and from PE was difficult under these conditions, possibly due to formation of various ionic forms of these phospholipids. This difficulty was resolved by performing HPLC with an ammonia-containing solvent and by equilibration of the sample in ammonia-containing solvent prior to injection.

Total ion current plot from HPLC–MS analysis of standard phospholipids is shown in **Fig. 6a.** The separation

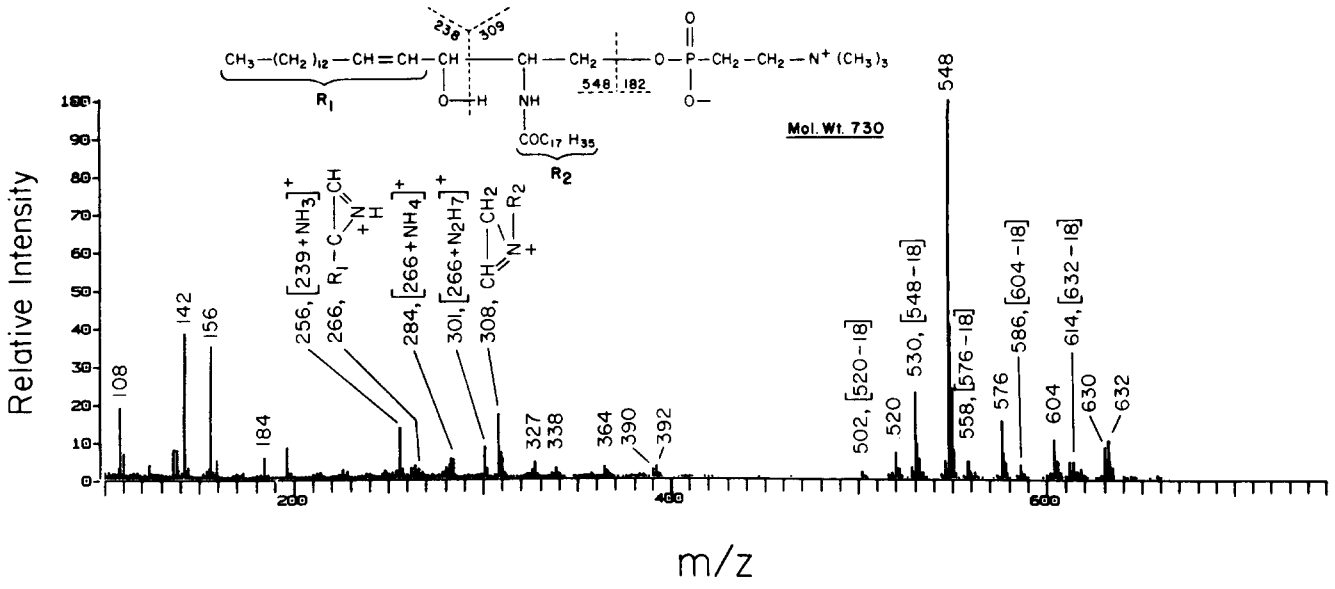
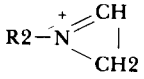


Fig. 5. Ammonia chemical ionization mass spectrum of bovine brain sphingomyelin (5 μg) in the positive ion mode. The spectrum was obtained as described in the legend of Fig. 1. Fragmentation of 18:0 fatty acid and C_{18}-sphingenine containing sphingomyelin is shown as an example. Ions m/z 364, 390, and 392 are with structure,  corresponding to 22:0, 24:1 and 24:0 fatty acid.

of phospholipids was achieved on a Brownlee silica gel cartridge column with ammonia-containing solvent as described in the experimental section. Under these conditions fairly good reproducibility was achieved. All the phospholipids were well resolved except PS which tailed to some extent into lysoPI peak. The specific ion plots for m/z 105, 141, 142, and 198 are given in Fig. 6, b–e. These ions are usually specific for the individual phospholipid bases. Thus m/z 105, specific for PS, is not found to be associated with other phospholipids. Similarly, m/z 141 is relatively specific for ethanolamine-containing phospholipids, whereas m/z 142 is specific for choline-containing phospholipids. M/z 198 is found to be associated only with inositol-containing phospholipids. HPLC–MS of phospholipids also shows that individual phospholipids were chromatographically resolved to some extent based upon molecular species. Thus, sphingomyelin was partially resolved into two peaks (Fig. 6d). Mass spectral analysis showed that the front peak contained mostly long chain fatty acid sphingomyelin such as 24:1, m/z 630 (Fig. 6h), whereas the later peak contained mostly short chain fatty acid-containing sphingomyelin such as 16:0, m/z 520 (Fig. 6f). Similarly, with respect to other phospholipids, the long chain fatty acid-containing species were eluted before the short chain fatty acid-containing species.

Total ion current plot after HPLC–MS analysis of rat brain phospholipids is given in **Fig. 7a.** The phospholipids were well resolved as can be seen by the specific ion plots for phospholipid bases (Fig. 7b–d). The ethanolamine-containing phsopholipids were partially resolved into two peaks (Fig. 7c). The mass spectral analysis of the earlier peak showed that it contained mostly alkenylacyl GPE, m/z 561, 587, and 589 corresponding to plasmalogens with 16:1–18:1 (33%), 18:1–18:2 (36%), and 18:2–18:2 (31%), respectively. The front peak, however, also contained some diacyl GPE, m/z 623, 627 and 651 (corresponding to 16:0–22:6 (20%), 18:0–20:4 (28%), and 18:0–22:6 (19%), respectively. The later eluting peak contained mostly diacyl GPE with 18:0–18:1 (m/z 605, 15%) and 18:0–18:2 (m/z 603, 19%). The measured ratio of alkenylacyl GPE to diacyl GPE was 58:42. These results agree well with the previously published analysis (13, 14).

Rat brain PC peak also split into two major peaks, the earlier peak contained mostly long chain fatty acid-containing species, whereas the latter contained short chain fatty acid species. From the relative intensity of the diglyceride ions of PC, it was calculated that, of the total rat brain PC, 5.4% was ether-containing PC, mostly 16:1–18:0 (m/z 563, 27%) and 18:0–20:4 (m/z 613, 25%) containing species. The major diacyl-containing PC's were: 16:0–18:1 (m/z 577, 43%), 16:0–16:0 (m/z 551, 19%); 18:0–18:1 (m/z 605, 11%); 16:0–18:0 (m/z 579, 9%); 16:0–16:1 (m/z 549, 6%); and 14:0–16:0 (m/z 523, 6%). Eight other minor species were also recognized in the mass spectrum. It is surprising to note that rat brain

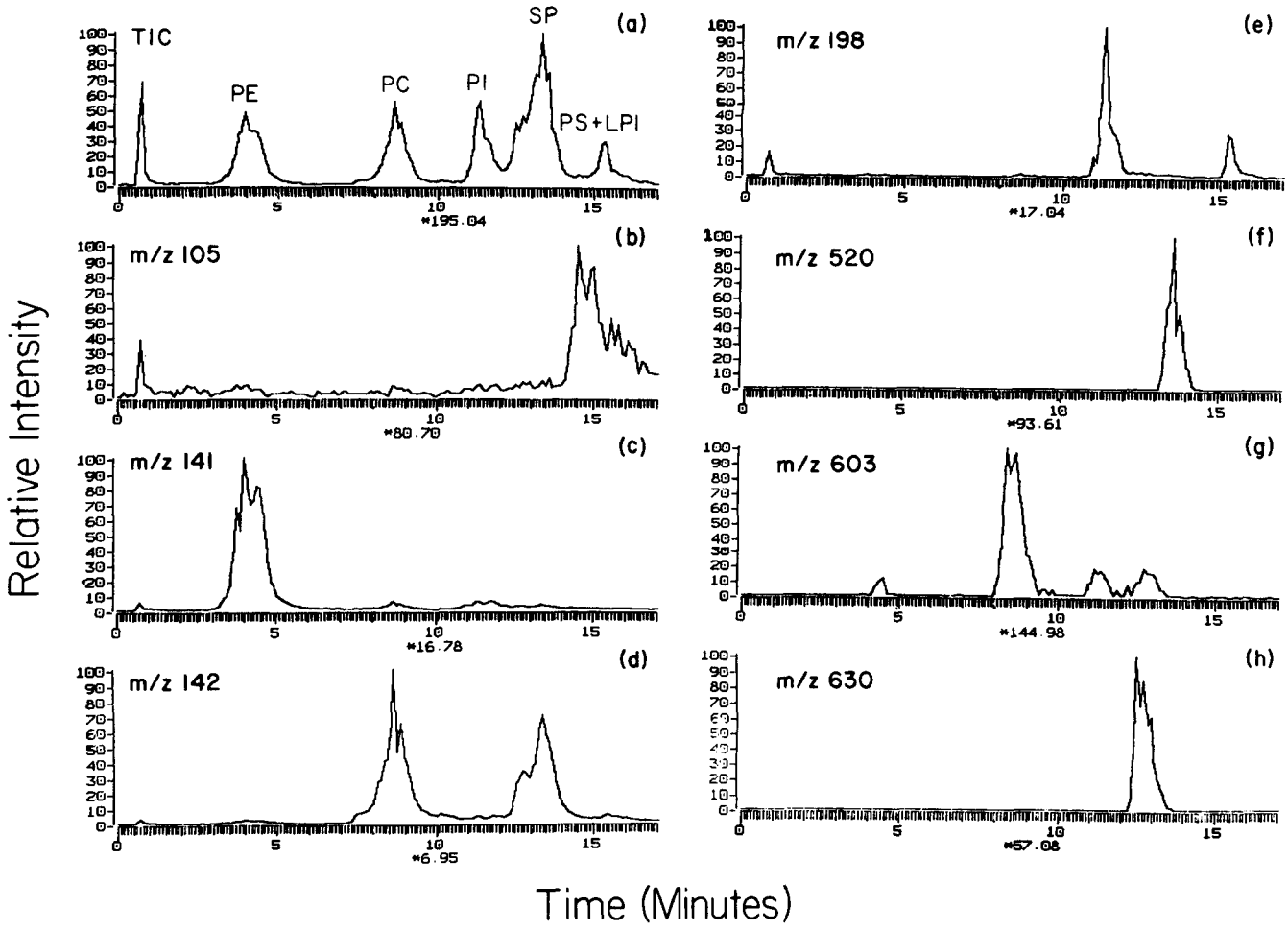


Fig. 6. Reconstructed plots of total ion current (a) and various specific ions (b–h) monitored after HPLC–CIMS of standard phospholipids. Bovine brain PE, PC, PS, PI, and SP (5 μg each) were injected on a Brownlee silica gel (5 μm) cartridge HPLC column and eluted as described in the text. The eluate was applied to the moving belt of a Finnigan HPLC–MS interface. The solvent was removed by heating the belt at 330°C under vacuum and the phospholipid was introduced into the ion source (150°C) of the mass spectrometer. Positive ion mass spectra were continuously collected in the chemical ionization mode with ammonia as the reagent gas from m/z 100–900, every 7 seconds, under the control of Teknivent model 56K MS data system. The magnification factor (*) is given under each panel. Lyso PI (LPI) is an iumpurity in PI sample.

PC contained several *bis*-saturated fatty acid-containing species in large amounts. Saturated species represented 35% of the total diacyl PC. Freysz and Van den Bosch (15) also reported, in a preliminary communication, high amounts of saturated fatty acid-containing PC in rat brain. The percentage composition of individual fatty acids in PC was calculated and found to be in good agreement with the previously reported amounts (16).

The amount of PI in rat brain phospholipids is very small (about 4%). The specific ion m/z 198, however, clearly identified the peak as PI. The relative intensity of the $[M + 35]^+ - 259$ ions indicated that the major species of rat brain PI were 18:0–20:4 (m/z 662 and 627, 90%); 18:0–20:3 (m/z 664, 5%); and 18:0–22:5 (m/z 688, 5%).

The rat brain SP was resolved into two major peaks. The front peak was associated mostly with SP with C_{18}-sphingenine and 22:0 (m/z 586 and 604, 4%); 24:1 (m/z 612 and 630, 12%); and 24:0 (m/z 614 and 632, 6%). The later peak contained SP with C_{18}-sphingenine and 16:0 (m/z 502 and 520, 5%); 18:0 (m/z 530 and 548, 54%); 20:0 (m/z 558 and 576, 5%) and C_{18}-sphinganine with 16:0 (m/z 522, 1%) and 18:0 (m/z 532 and 550, 10%). Small amounts (<3%) of other minor species of SP were also identified.

The last peak in the chromatogram was due to PS (m/z 105). The major molecular species of rat brain PS was identified as 18:0–18:1 fatty acid containing PS.

DISCUSSION

In the past few years significant progress has been made in overcoming technical problems associated with inter-

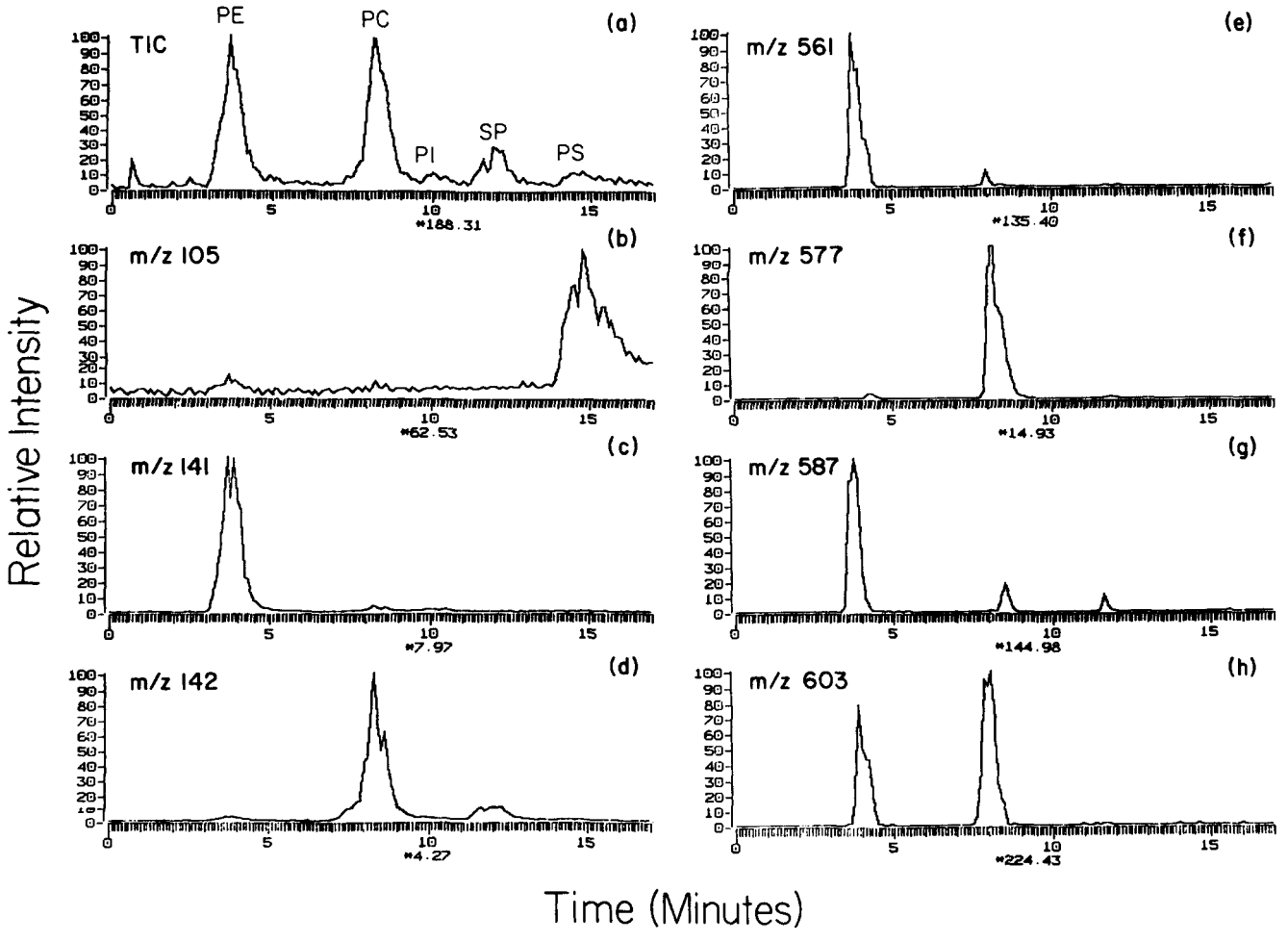


Fig. 7. *Reconstructed plots of the total ion current (a) and various specific ions (b–h) monitored after HPLC–CIMS of rat brain phospholipids* (80 μg). HPLC–CIMS data were collected as described in the legend of Fig. 6.

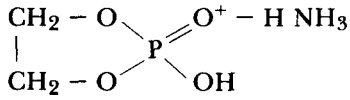
facing of HPLC to MS. An "on-line" HPLC–MS system is preferred which is capable of analyzing compounds eluted as sharp narrow peaks without loss of resolution. A transport-type of interface has been found quite suitable for removal of HPLC solvent and introduction of the residue into mass spectrometer.

HPLC–CIMS provides a new powerful technique for the analysis of lipids. We have previously reported the use of HPLC–MS for the analysis of sphingoid bases obtained from complex sphingolipids (5). Here we have described the potential use of this technique for the direct analysis of phospholipids. Privett and Erdahl (17) have previously reported coupling of liquid chromatography to mass spectrometry for the analysis of lipids with a moving-wire transport. However, in their system only 15% of the eluted sample was analyzed by MS. Also, the sample had to be converted to hydrocarbons in a reactor by hydrogenolysis and reductive cracking by reaction with H_2 in the carrier gas catalyzed by the nickel in the moving belt. The largest ions reported in their spectra of phospholipids were for free fatty acids, thus the molecular species information residing in the diglyceride ions was completely lost.

Klein (18) has previously reported electron ionization MS of several intact phosphatidylcholines. A few reports have also appeared on the analysis of phospholipids by field desorption and fast atom bombardment mass spectrometry (19–22). Isobutane chemical ionization mass spectrometry of dioleoyl-GPC has been previously reported by Foltz (23), whereas positive ion-ammonia CIMS of synthetic phosphatidylcholines has been reported by Crawford and Plattner (24). Here we have described positive and negative CIMS of several other naturally occurring phospholipids besides PC with ammonia and methane as the reagent gases.

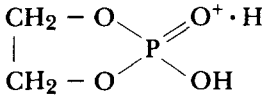
Structural characterization of various ions produced in the CIMS of various phospholipids is only briefly discussed here (Table 1) and will be described in detail elsewhere. Although some of the ion structures of fragments of PC in the positive ion-ammonia CIMS have been pro-

posed by Crawford and Plattner (24), ambiguity remains, based on our results. For example, the positive ion mass spectra of PC obtained with ammonia and methane as the reagent gases were exactly the same in the high mass range although the intensities were different. If the ion structure $[M + NH_4]^+ - N(CH_3)_3$ for $M^+ - 41$ ion as proposed by Crawford and Plattner (24) is correct, then it should not be present in the methane spectra. Again, they have described $[M + 35]^+ - 182$ ion as the ammonia adduct; however the same ion was found in the methane spectra of PC although with lower intensity (Fig. 1 A, B). We analyzed synthetic PC with CD_4 as the reagent gas; in these spectra the high mass unit ions were $[M + 1]^+$, $M^+ + 2$, $[M + 33]^+ - 182$, and $M^+ - 182$. These data do not support the structures of these fragments of PC as proposed by Crawford and Plattner (24) and more work is necessary to completely define the ion-structures of the fragments. In the positive ion-ammonia CIMS of PC, the strongest ion was at m/z 142 in the low mass range. The possible structure (24) for this ion derived from phosphocholine could be

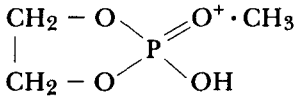


m/z 142.

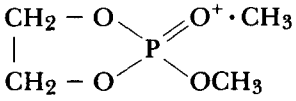
In the positive ion methane CIMS of PC, the major ions were at m/z 125, 139, and 153 with the possible structures:



m/z 125

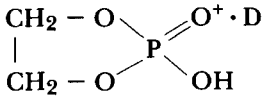


m/z 139

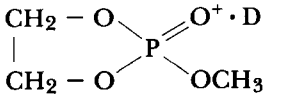


m/z 153

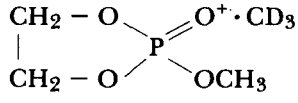
In the CD_4 positive ions CIMS of PC these ions were at m/z 126, 140, and 156 with possible structures:



m/z 126



m/z 140



m/z 156

CD_4 CIMS spectral data thus support the above proposed ion structures.

In the positive ion-ammonia or -methane CIMS of other phospholipids such as PE, PS, PI, SP, and PG, the diglyceride or the ceramide ion is formed by the cleavage of the phosphobase. In the positive ion-methane CIMS, $[M + 35]^+ -$ phosphobase and $[M + 17]^+ -$ phosphobase ions are of lower intensity than those obtained with ammonia as the reagent gas (Fig. 1A and B) for all phospholipids. Thus the major ion is the diglyceride ion in the high mass range for the glycerophospholipids, which simplifies the calculations for the relative abundance of the major molecular species. However, the characteristic ion of the phospholipid base is not of high intensity with methane as the reagent gas and therefore it is not useful for HPLC monitoring, if high sensitivity is desired.

The percentage distribution of molecular species of various phospholipids as determined by mass spectrometry agreed fairly well with that obtained by other methods. These results indicate that mass spectrometry has good potential for direct analysis of molecular species of phospholipids. Further work is necessary with internal standards to confirm the accuracy of quantitative analysis.

Coupling of MS with HPLC offers an important advance in analytical methodology. The mass spectrometer not only functions as a highly sensitive universal detector but also serves to provide valuable structural information not obtainable otherwise on a small quantity of compounds. Previously, we and others have demonstrated that moving-belt interface works well for such a purpose and that peak-broadening is minimal (5, 25) with appropriate solvent application techniques. Although theoretically any solvent could be used for HPLC analysis in this system, practical considerations would require that solvents with high volatility be preferred to facilitate evaporation from the belt. Naturally occurring phospholipids are a complex mixture of compounds having similar polarity. Thus separation of phospholipids on a chromatographic column is difficult. Separation is also hampered by the existance of various salt forms of the same phospholipids or a mixture of salts and free bases. In our experience the HPLC of phospholipids on a silica column is best achieved by the use of solvents containing ammonia. The same separation is consistently obtained providing the solvent is well equilibrated with the column. Most of the commonly occurring phospholipids are well resolved. The separation could be improved, however, especially for PS (which tails in this system) and to accommodate the resolution of other minor phospholipids in natural mixtures, such as PA, cardiolipin, and lysophospholipids.

Quantitative analysis of individual phospholipids by HPLC–MS technique is not addressed in this study. However, this should not be difficult since each phospholipid produces specific ions in low mass range. Also, we have not explored the limits of sensitivity of this system. How-

ever, we have routinely injected about 5 μg of individual phospholipid for complete HPLC–MS analysis. The sensitivity can easily be increased fivefold to obtain reliable information on the molecular species of individual phospholipids. If one is just interested in knowing the amount of phospholipid in a mixture, specific ion monitoring in the low mass range for an individual phospholipid should provide detection capability at a subnanogram level.

In summary, HPLC–CIMS overcomes the limitations of the detection method encountered in HPLC. Simultaneously, the method provides extensive information on the molecular structure of each phospholipid and on the relative abundance of each molecular species of an individual class of phospholipids. Such information is obtained by this method with only a few μg of the lipids in a few minutes. By conventional methods, analysis of this type would require separation of large quantities of individual phospholipids by TLC or by another chromatographic method, followed by enzymatic and/or chemical degradation of the individual phospholipids and GLC analysis of the liberated diglycerides or individual fatty acids.

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