papers and notes on methodology

Separation of phospholipids and individual molecular species of phospholipids by high-performance liquid chromatography

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Abstract Isocratic high-performance liquid chromatography methods are described for separating the major classes of phospholipids and for isolating the individual molecular species of phospholipids. Fractionation of a total lipid extract of rat liver on a silica column resulted in quantitative recoveries of all major phospholipids with preservation of their fatty acid composition. Rat liver phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine were then each chromatographed on a C18 reverse phase column to isolate individual molecular species. Component peaks were identified by their fatty acid composition and quantitated by phosphorus determination. Using this method we found that for each of these phospholipids from 30 to 35 different molecular species can be routinely identified and reproducibly quantitated. A characteristic elution sequence of molecular species permitted their identification based upon their retention times on a reverse phase column.—**Patton, G. M., J. M. Fasulo, and S. J. Robins.** Separation of phospholipids and individual molecular species of phospholipids by high-performance liquid chromatography. *J. Lipid Res.* 1982. **23:** 190–196.

Supplementary key words phosphatidylcholine • phosphatidylethanolamine • phosphatidylinositol • phosphatidylserine

As currently performed, the separation of phospholipids into individual molecular species is a complex, multistep procedure. First, phospholipid classes must be quantitatively isolated in amounts large enough for further analysis. This fractionation has traditionally been undertaken by some form of column chromatography which, notwithstanding the use of a great variety of stationary phases and solvent elution procedures (1, 2), often results in imperfect resolutions and incomplete recoveries. However, once a pure phospholipid fraction is obtained, its molecular components can be identified (3–6). The analysis of molecular species has customarily been performed in a series of steps which include partial hydrolysis, derivative formation, and/or a combination of several different types of chromatographic procedures (see references 7 and 8 for reviews of these methods).

Recently, HPLC methods have been introduced to separate phospholipids (9–19). However, separation of all classes of phospholipid has not as yet been accomplished, and, thus far, there have been only two reports (15, 16) in which molecular species separations have been attempted. Moreover, in only one of these studies, one in which SPH has been fractionated, was it demonstrated that it is possible to separate the multiple molecular species of a phospholipid from a biologic source (16).

We have modified one HPLC method (12) to separate, for the first time, all major phospholipid classes. Using rat liver, we have demonstrated that recoveries of phospholipids are complete and that the molecular composition of the fractions isolated is not changed by this chromatography. In addition, we have developed reverse phase HPLC procedures which effectively separate and permit predictable identification of the molecular species of PC, PE, PI, and PS.

Abbreviations: HPLC, high-performance liquid chromatography; NL, neutral lipids; PE, phosphatidylethanolamine; PA, phosphatidic acid; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; PC, phosphatidylcholine; SPH, sphingomyelin; LPC, lysophosphatidylcholine; RRT, relative retention time.

MATERIALS AND METHODS

Lipids were extracted from rat liver by the method of Folch, Lees, and Sloane Stanley (20). The washed extract was dried under N_2 and redissolved in hexane–2-propanol–water 40:54:6 for HPLC of phospholipids. Chromatography was performed with a Waters Associates (Milford, MA) Model 6000A or Model M-45 solvent delivery system equipped with a Model U6K injector and a Model 450 variable wavelength detector which was operated at 205 nm. The lipid extract was separated into phospholipid classes by chromatography on a 250 × 4.6 mm Hibar II column packed with 10 μ LiChrospher Si-100 (EM Laboratories, Inc., Elmsford, NY). The eluting solvent was prepared by the addition of 490 ml of HPLC-grade 2-propanol to 62 ml of 25 mM potassium phosphate (pH 7.0). After mixing, 367 ml of HPLC-grade hexane and 100 ml of absolute ethanol were added. The solvent was filtered through a 0.5 μ type FH Millipore filter (Millipore Corp., Bedford, MA), and then 0.6 ml of glacial acetic acid was added. The final solvent composition is hexane–2-propanol–25 mM phosphate buffer–ethanol–acetic acid 367:490:62:100:0.6.

PC, PE, PI, and PS were separated into molecular species on a 4.6 × 250 mm Ultrasphere ODS column (Altex Scientific, Inc., Berkeley, CA). PC, PE, and PI were eluted with 20 mM choline chloride in methanol–water–acetonitrile 90.5:7:2.5, at a flow rate of 2.0 ml/min. PS was eluted with 30 mM choline chloride in methanol–25 mM KH_2PO_4–acetonitrile–acetic acid 90.5:7:2.5:0.8, also at 2.0 ml/min. PC and PE were applied to the column in 50–100 μl of ethanol, and PI and PS in 10–20 μl of chloroform.

Fractions collected from the column were dried under a stream of N_2 and re-extracted (20). Aliquots were then taken for total lipid phosphorus, fatty acid analysis, and two-dimensional paper chromatography for identification of individual phospholipid classes. Paper chromatography and phosphorus analysis were performed as described by Wuthier (21). Fatty acids were determined by capillary gas–liquid chromatography after methylation with 2% HCl in methanol. Chromatography was performed on a 50-m Silar 5 column with 17:0 as an internal standard as described by Patton et al. (22).

HPLC-grade hexane and 2-propanol were obtained from Waters Associates, absolute ethanol from Publicker Industries (Linfield, PA), silica gel-impregnated paper (SG-81) from Whatman, Inc., (Clifton, NJ) and choline chloride from General Biochemicals (Chagrin Falls, OH). HPLC-grade methanol, HPLC-grade acetonitrile, and other solvents and chemicals, which were of reagent grade purity, were obtained from Fisher Scientific (Medford, MA).

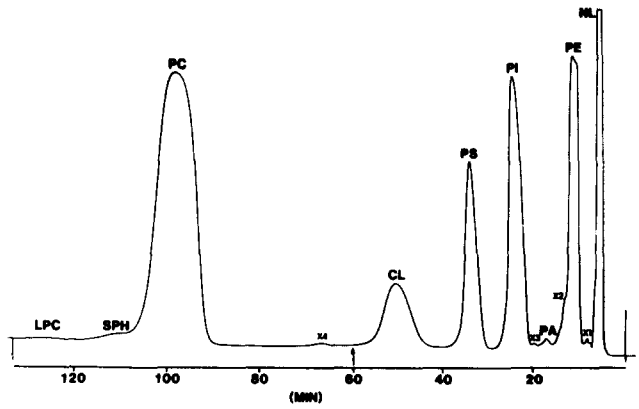

Fig. 1. Separation of phospholipid classes by HPLC. A total lipid extract of rat liver, containing 60 μg of lipid phosphorus (which corresponds to approximately 1.5 mg of phospholipid) was chromatographed as described in Materials and Methods. The flow rate was 0.5 ml/min at the start and then changed to 1 ml/min as shown by the arrow. Detection was by absorption at 205 nm (2.0 absorbance units full scale).

RESULTS

Separation of phospholipid classes

Fig. 1 shows a chromatogram of a total lipid extract of rat liver. Chromatography was regularly completed in about 2 hr by changing the flow rate as indicated. The collected fractions were identified by two-dimensional paper chromatography (21). Complete separation was achieved for all major phospholipid classes with one exception, the separation of PC from SPH. In three determinations, an average of 4.6 ± 0.7 (SD)% of the PC was contained in the SPH fraction and 23.9 ± 8.0% of the SPH was with the PC.

Several small peaks (labeled X1, X2, X3, and X4) were regularly detected but were not identified. X1 contained no phosphorus and 0.2% of the total fatty acids. X2, which appeared as a shoulder on the back of the PE peak, may represent another molecular form of PE since only a single PE spot was detected by two-dimensional chromatography. Neither X3 nor X4 were present in amounts large enough to characterize.

The percentage of individual phospholipids determined by phosphorus analysis after HPLC was compared with the percentage determined by phosphorus analysis after paper chromatography (**Table 1**). Small differences were noted for only SPH and PE. Although we have no explanation for the observed discrepancy in PE determination, the differences in SPH may have been due to some loss of SPH into the PC fraction with HPLC. Recovery of total lipid phosphorus was essentially complete with both methods, and averaged 96.9 ± 5.3 (SD)% for HPLC and 98.4 ± 4.1% (n = 3) for paper chromatography.

TABLE 1. Phospholipid composition of rat liver as determined after HPLC and after two-dimensional paper chromatography

Phospholipid Fraction	HPLC	Two-dimensional Chromatography
	% (±SD) of lipid phosphorus[a]	
PE	25.76 ± 0.42	24.41 ± 0.29[c]
PA	0.39 ± 0.18	0.42 ± 0.22
PI	6.90 ± 0.28	6.74 ± 0.27
PS	2.75 ± 0.22	3.14 ± 0.15
CL	4.60 ± 0.23	4.84 ± 0.05
PC	54.63 ± 0.79	54.84 ± 0.44
SPH	3.20 ± 0.25	3.93 ± 0.08[c]
LPC	0.91 ± 0.28	0.79 ± 0.09
Unidentified[b]	0.86 ± 0.20	0.89 ± 0.23

[a] Results are shown as the mean ± SD for three determinations.
[b] The unidentified fraction of HPLC represents the sum of the collected effluents between peaks (including X1 through X4 in Fig. 1). The unidentified fraction of two-dimensional chromatography represents a single area near CL which was not further characterized.
[c] $P < 0.05$, as calculated by Student's t test.

The fatty acid composition of individual phospholipids is compared to the fatty acid composition of the unchromatographed sample in **Table 2.** Values shown are for those fatty acids which are present in amounts greater than 0.5 μmol/g liver. For each fatty acid, the sum of its concentration in the individual fractions is similar to its concentration in the unfractionated extract. In particular, we found no selective loss of unsaturated fatty acids as a result of HPLC.

Separation of phospholipids into molecular species

Fig. 2 shows chromatograms of the separations of rat liver PC, PE, PI, and PS into individual molecular species. The composition of each collected peak was determined by fatty acid analysis (22). Identification of peak components is shown in **Table 3.** The position of the fatty acids in each molecular species was based upon the detailed analysis of rat liver lecithins that have been reported by other investigators (summarized in reference 8). We found that all four phospholipid classes that were studied contain essentially the same molecular species, although the distribution of species is clearly different in each class (Fig. 2). As shown in Table 3, molecular species separations occur even on the basis of the position of double bonds. It was found that molecules with 22:5 (n-3) elute earlier than otherwise similar molecules that contain 22:5 (n-6). A similar effect was observed for molecules containing 20:3 (n-6) and (n-9) fatty acids. It is probable that positional isomers of the mono- and diunsaturated fatty acids also elute at different times. However, with the efficiency of the column used, this difference was not evident. The percent distribution of the molecular components, as determined by phosphorus analysis, is shown in Table 4. Since many of the molecular species of PI and PS were present only in trace amounts in the sample of liver analyzed, it was necessary to collect them in batches to have sufficient material for analysis. Recoveries, as determined by phosphorus measurement, were near 100% for each of the phospholipids analyzed (Table 4).

The recovery of isolated fractions was further assessed for both PC and PE by a comparison of the fatty acid composition of the unchromatographed with the chromatographed sample. As shown in **Table 5,** values determined after HPLC closely approximate values directly determined by gas–liquid chromatography of the fatty acids in the original, unfractionated sample. This comparison was, however, not undertaken for either PI or PS, in which a large number of individual peaks were collected together for quantitation.

TABLE 2. Fatty acid composition of rat liver after HPLC (μmol/g wet weight of liver)

	HPLC Fractions											
Fatty Acid[a]	NL	PE	PA	PI	PS	CL	PC	SPH	LPC	Rest[b]	Total	Original Sample
16:0	3.22	2.82	0.15	0.22	0.09	0.08	7.25	0.32	0.09	0.16	14.40	13.49
16:1 (n-7)	0.16	0.04	0.01	0.01		0.14	0.16	0.02		0.01	0.56	0.46
18:0	0.65	3.99	0.11	2.05	0.85	0.03	8.21	0.06	0.03	0.06	16.04	15.14
18:1 (n-9)	1.95	0.47	0.04	0.06	0.05	0.13	1.77	0.04	0.02	0.06	4.59	4.39
18:1 (n-7)	0.43	0.20	0.05	0.01	0.01	0.30	0.60	0.02		0.01	1.63	1.38
18:2 (n-6)	1.44	0.98	0.04	0.06	0.04	2.24	3.83	0.12	0.01	0.07	8.83	8.62
20:3 (n-6)	0.03	0.08		0.08	0.01	0.07	0.29				0.56	0.57
20:4 (n-6)	0.54	4.02	0.05	1.75	0.41	0.04	7.99	0.04	0.02	0.02	14.68	14.79
22:5 (n-3)	0.07	0.21		0.03	0.02		0.19				0.52	0.52
22:6 (n-3)	0.26	1.45	0.02	0.06	0.18	0.02	1.57				3.56	3.89
Remainder	1.16	0.63	0.03	0.22	0.07	0.08	0.88	0.16	0.10	0.08	3.41	2.31
Total	9.37	14.85	0.51	4.55	1.73	3.13	32.54	0.76	0.27	0.47	68.78	65.56

[a] Only individual fatty acids present in amounts > 0.5 μmol/g wet weight are shown.
[b] Rest = fatty acids between major peaks.

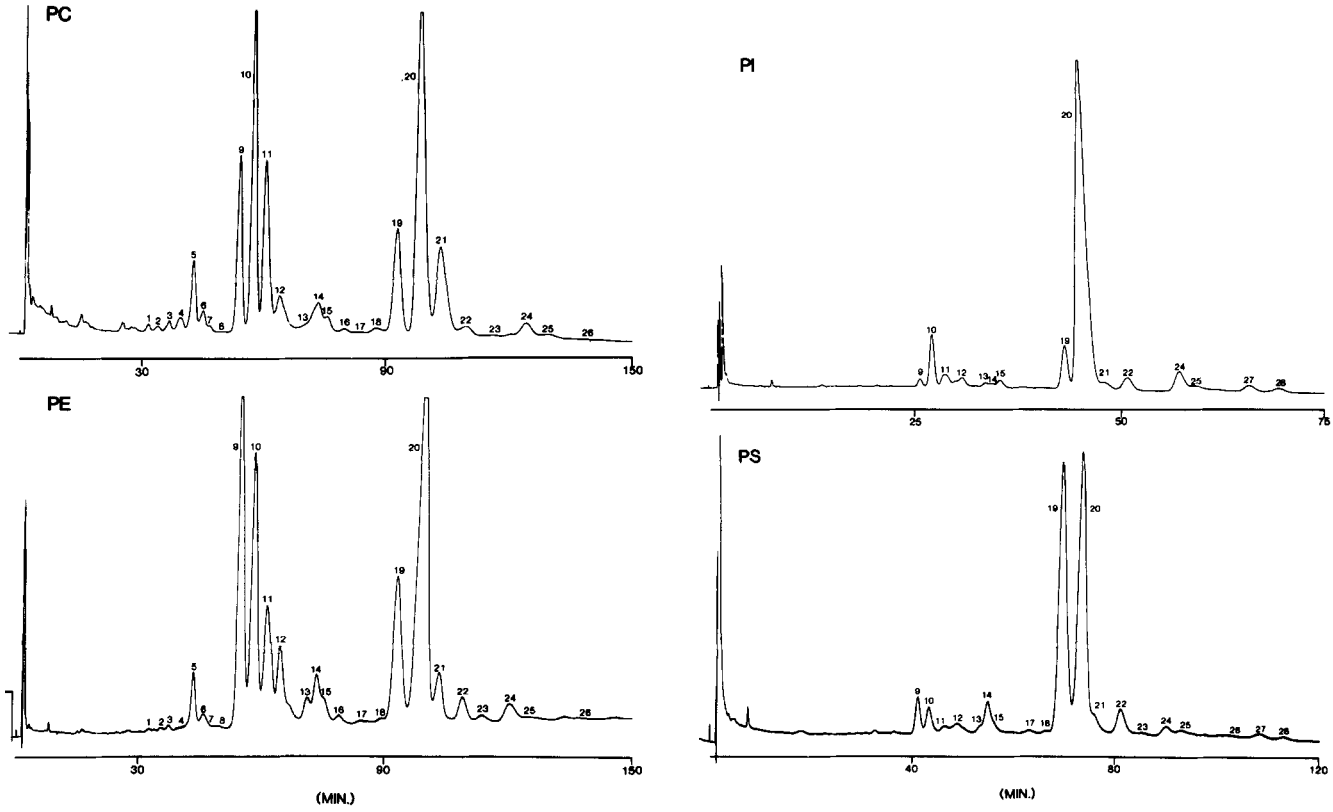

Fig. 2. HPLC separation of the molecular species of rat liver PC, PE, PI, and PS. In the examples shown, 220 μg of PC, 224 μg of PE, 48 μg of PI, and 55 μg of PS were chromatographed on a C18 reverse phase column. The column was eluted as described in Materials and Methods. Detection was by absorption at 205 nm (0.2 absorbance units full scale). Peaks are numbered in sequence of elution and are identified in Table 3. PI and PS, peaks which were too small to be detected in the examples shown, were not numbered.

DISCUSSION

HPLC separation of phospholipid classes

We have described an improved HPLC method which is capable of effectively separating the major classes of phospholipids without the use of gradient elution. Using normal rat liver, we have found that this procedure requires no preliminary separation of phospholipids from other lipid components and can be performed with up to 2.5 mg of phospholipid which can be quantitatively recovered after fractionation. We have further shown by fatty acid analysis that this chromatography does not result in any appreciable change in phospholipid composition. A major advantage of the current procedure is the complete separation of PS, PI, and CL fractions. To our knowledge, this has not been previously accomplished by HPLC. Although the solvent system used in the present study contains a number of components, we have found that the discrete separation of PS, CL, and PI largely depends on the concentration of acetic acid. With the particular LiChrospher Si-100 column used, 0.06% acetic acid produced optimum separations. However, when using other LiChrospher Si-100 columns, we have found that as much as 0.08% or as little as 0.035% acetic acid was required to produce the same results.

TABLE 3. Molecular species of rat liver phospholipids separated by HPLC

Peak[a]	Molecular Species[b]	Peak	Molecular Species
1	14:0–22:6	15	16:0–20:3 (n-6)
2	18:2–18:3; 14:0–20:4	16	17:0–18:2; 16:0–20:3 (n-9)
3	16:1–18:2	17	16:0–18:1
4	18:2–22:6; 15:0–22:6	18	18:1–18:1
5	16:0–20:5; 18:2–20:4	19	18:0–22:6
6	18:2–18:2	20	18:0–20:4
7	16:0–16:1	21	18:0–18:2
8	16:1–18:1	22	18:0–17:1; 17:0–18:1
9	16:0–22:6	23[c]	18:0–22:5 (n-3)
10	16:0–20:4	24	18:0–22:5 (n-6)
11	16:0–18:2	25	18:0–20:3 (n-6)
12	18:1–18:2	26	18:0–18:1
13	16:0–22:5 (n-3)	27[d]	20:0–22:6
14	16:0–22:5 (n-6); 18:0–20:5	28[d]	20:0–20:4

[a] Peak numbers correspond to the elution sequence shown in Fig. 2.

[b] The fatty acids in each peak were identified by gas–liquid chromatography (22). Molecules are shown as the fatty acid in position 1-fatty acid in position 2.

[c] This peak contains more lipid phosphorus than can be accounted for by the fatty acid amounts.

[d] Peaks were identified by their retention time, and not by gas–liquid chromatography.

TABLE 4. Distribution of molecular species of rat liver phospholipids (%)

Peak Number	PC	PE	PI	PS
1	0.62 ± 0.12			
2	0.70 ± 0.18	1.35 ± 0.22		
3	1.14 ± 0.49			
4	1.23 ± 0.27		2.51 ± 0.84	0.97 ± 0.48
5	2.07 ± 0.38	1.97 ± 0.18		
6	1.48 ± 0.53	1.38 ± 0.54		
7	0.69 ± 0.45			
8	0.98 ± 0.47	0.81 ± 0.49		
9	3.90 ± 0.24	9.03 ± 0.66		
10	9.66 ± 0.48	10.65 ± 1.26	4.65 ± 0.59	6.06 ± 1.72
11	14.41 ± 0.29	12.19 ± 0.79	5.13 ± 0.22	
12	3.77 ± 0.20	6.05 ± 0.16		
13	1.00 ± 0.18	1.60 ± 0.21		
14	2.62 ± 0.31		3.04 ± 0.68	
15	1.30 ± 0.22	4.03 ± 0.02		4.07 ± 0.64
16	1.48 ± 0.25			
17	1.41 ± 0.19	1.22 ± 0.44	1.95 ± 0.22	1.39 ± 0.77
18	6.35 ± 0.57	2.23 ± 0.46		
19	3.58 ± 0.26	7.72 ± 0.11	3.06 ± 1.24	24.84 ± 1.75
20	16.06 ± 0.48	24.52 ± 1.03	57.83 ± 1.66	39.49 ± 3.50
21	14.81 ± 0.13	6.83 ± 0.50		4.99 ± 1.32
22	1.12 ± 0.35	2.35 ± 0.50	5.48 ± 0.39	5.02 ± 0.58
23	3.26 ± 1.05	0.86 ± 0.20		
24	1.41 ± 0.38	2.78 ± 0.22	8.50 ± 1.43	6.01 ± 1.13
25	1.56 ± 0.09			
26	3.43 ± 0.68	2.87 ± 0.78	5.81 ± 1.01	5.49 ± 1.10
27			2.04 ± 0.38	1.68 ± 0.71
Recovery	92.8 ± 6.8	105.2 ± 4.7	94.6 ± 1.7	96.7 ± 15.7

Results are shown as the mean ± SD. HPLC was performed in triplicate for each of the PC, PE, PI, and PS fractions isolated from a single rat liver. Percentages of peaks and recoveries were determined by phosphorus analysis (see Methods). Adjacent peaks containing relatively small amounts in a sample were collected together for analysis and are shown within brackets.

The only apparent limitation of this procedure is the incomplete separation of PC from SPH. However, if necessary, PC and SPH may be readily resolved by rechromatographing the collected fractions in a second system (10, 17, 19).

HPLC separations of molecular species of PC, PE, PI, and PS

We have developed HPLC systems which for the first time permit direct separation and identification of the molecular species of PC, PE, PI, and PS. Although a limited number of synthetic phosphatidylcholines have previously been fractionated by HPLC (15), this appears to be the first reported fractionation of naturally occurring PC.[1] Of the major phospholipids, only SPH has been fully fractionated by HPLC (16) and, therefore, was not reexamined in the current work.

The HPLC procedures described permit quantitative recoveries of the individual molecular components as demonstrated for the PC, PE, PI, and PS classes that were isolated from rat liver (Table 4). The current reverse phase HPLC procedure permitted good separations for quantitation by phosphorus analysis using as little as 200 μg and as much as 1000 μg of either PC or PE. However, because of the marked predominance of only one or two species in the PI and PS fractions of liver, a much smaller sample size was required for chromatography. Optimum separations for quantitation could be achieved with from 50 μg of either PI or PS to 300 μg of PI and 400 μg of PS. The use of larger samples for either of these phospholipids resulted in obliteration of the small peaks adjacent to the predominant peaks and, additionally, resulted in tailing of other components. However, if only qualitative information is desired, as little as 10 μg of PC or PE, and 2 to 3 μg of PI or PS can be used.

The retention time of the molecular species of phospholipids depends both on the polar head group and on the fatty acid composition of the particular molecule. Of

[1] Since submission of this manuscript, separation by HPLC of the molecular species of PC has also been reported by M. Smith and F. B. Jungalwala (1981. J. Lipid Res. 22: 697–704).

[2] The RRT is obtained by dividing the retention time of each peak by the retention time of a reference peak.

TABLE 5. Comparison of the fatty acid composition of PC and PE as determined by direct analysis of the original sample[a] and after HPLC[b]

Fatty Acid	% in PC		% in PE	
	Original	After HPLC	Original	After HPLC
16:0	18.58	17.14	20.32	18.82
16:1	1.39	1.90	0.81	0.65
18:0	24.40	23.05	24.52	24.16
18:1	9.11	11.14	7.39	7.79
18:2	17.71	19.54	11.13	13.60
20:3	2.10	1.87	1.29	1.89
20:4	16.72	18.56	21.00	18.08
20:5	0.96	0.95	1.60	1.68
22:5	1.47	2.14	1.53	1.53
22:6	4.71	4.67	7.24	8.72
Remainder	2.91		3.11	

[a] The distribution was determined by gas–liquid chromatography of the fatty acids present in the whole sample of PC and PE before reverse phase HPLC.

[b] The fatty acid distribution was calculated from the molecular species identifications in Table 3 and the % distribution of molecular species shown in Table 4. All of the fractions in which a particular fatty acid was present were totalled and were then divided by 2 (i.e., divided by one of the two fatty acids present in the molecule). For the purposes of this calculation, it was assumed that when more than one molecular component was present in a peak, the components were present in equal amounts.

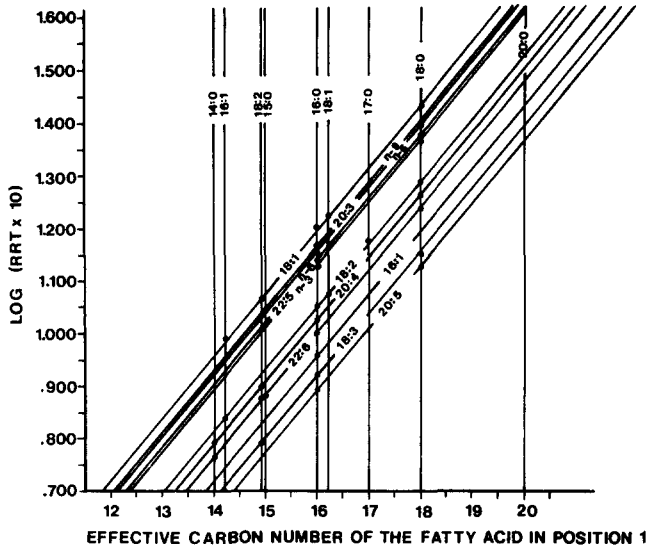

Fig. 3. The effect of fatty acid composition on the elution pattern of molecular species of phospholipids. The RRT calculated for each molecule (Table 5) was plotted as the log of the RRT × 10 against the carbon number of the fatty acid in position-1 (as described in the Discussion). The points represent the molecular species identified by gas–liquid chromatography (Table 3). Oblique lines connect molecules that have the same fatty acids in position-1. The intersection of oblique and perpendicular lines provides the RRT of individual molecules.

the phospholipids studied, PI eluted most rapidly, followed by PS, and then PC and PE (Fig. 2). However, within any class of phospholipid, the order of elution of molecular species was constant and entirely dependent on the composition of the component fatty acids. That is, the relative retention time[2] of any particular molecular species was the same in all of the phospholipid classes studied. The RRTs of the major molecular species of phospholipids identified in rat liver are shown in **Table 6.**

TABLE 6. Relative retention times of the molecular species of PC, PE, PI, and PS in rat liver

Molecular Species	RRT[a]	Molecular Species	RRT
14:0–22:6	0.579	16:0–22:5 (n-6)	1.355
14:0–20:4	0.621	18:0–20:5	1.355
18:2–18.3	0.621	16:0–20:3 (n-6)	1.432
16:1–18:2	0.687	16:0–20:3 (n-9)	1.470
18:2–22:6	0.759	17:0–18:2	1.470
15:0–22:6	0.759	16:0–18:1	1.549
16:0–20:5	0.791	18:1–18:1	1.644
18:2–20:4	0.791	18:0–22:6	1.738
18:2–18:2	0.834	18:0–20:4	1.841
16:0–16:1	0.895	18:0–18:2	1.941
16:1–18:1	0.972	17:0–18:1	2.066
16:0–22:6	1.000	18:0–17:1	2.066
16:0–20:4	1.059	18:0–22:5 (n-3)	2.251
16:0–18:2	1.117	18:0–22:5 (n-6)	2.355
18:2–18:1	1.156	18:0–20:3 (n-6)	2.495
16:0–22:5 (n-3)	1.317	18:0–18:1	2.679

[a] For this calculation 16:0–22:6 was used as the reference peak and the RRT of all other peaks was then determined by dividing the retention time of each peak by the retention time of 16:0–22:6.

Using the RRT, it is possible to develop a graphic relationship that demonstrates that molecular species elute in a predictable sequence. This relationship, which is shown in **Fig. 3,** was constructed in the following manner for those molecular forms found in the liver. First, for molecules with a saturated fatty acid in position-1 and an unsaturated fatty acid in position-2, the carbon number of the fatty acid in position-1 was plotted on the x-axis against the RRT of the whole molecule on the y-axis (or, actually as plotted, the log of RRT × 10). A line was then drawn that connected the points of those molecules which have the same fatty acid in position-2, and a series of oblique parallel lines were obtained. Second, to plot molecules with an unsaturated fatty acid in both positions 1 and 2, the RRTs of those molecules were plotted on the oblique line for the fatty acid in position-2. A line was then drawn through this point parallel to the y-axis. The x-axis intercept of this line then provided the effective carbon number for the unsaturated fatty acid in position-1. For example, to determine the effective carbon number of 18:2 in position-1, one would plot the RRT for a molecule such as 18:2–20:4 on the oblique line of 20:4. A line from that point parallel to the y-axis would be drawn to intersect the x-axis. The intercept on the x-axis, which in this case was calculated as 14.93, is then the effective carbon number of 18:2 in position-1. To determine the RRT

of any molecular species, one would simply find the intercept of the line for the fatty acid in position-1 with the oblique line for the fatty acid in position-2.

Calculation of effective carbon numbers for other unsaturated fatty acids demonstrated that there is probably a constant relationship for each homologous series of fatty acid. That is, the effective carbon number of 16:1 (in position-1) is 14.22 or exactly two carbon units less than 18:1, which is 16.22. It is likely that all of the monounsaturates have a similar relationship to each other, and that the diunsaturates, triunsaturates, etc. also have a constant relationship with each other. Thus, since 18:2 (n-6) in position-1 has an effective carbon number of 14.93, it is probable that 20:2 (n-6) in position-1 would have a value of 16.93.

A constant pattern based on carbon number was also evident for the fatty acids in position-2. That is, the 16:1 (position-2) line is 2 carbon units less than the 18:1 (position-2) line. The same is true for the 20:5 (n-3) and 22:5 (n-3) lines, and presumably is also true for all other homologous series, i.e., 20:4 (n-6)/22:4 (n-6); 18:3 (n-3)/20:3); etc. Thus, it is possible to predict the effective carbon number (for fatty acids located in position-1) as well as the position of the line for unsaturated fatty acids in position-2 for a large number of molecules which have not actually been identified in this paper.

In conclusion, we have presented HPLC methods for the direct identification of phospholipid classes and the individual molecular species of PC, PE, PI, and PS. We have found that these methods permit reproducible identification and quantitative analysis of a diversity of phospholipids isolated from a biologic source.

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