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COMBINED ANALYSIS OF PHOSPHOLIPIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THIN-LAYER CHROMATOGRAPHY

ANALYSIS OF PHOSPHOLIPID CLASSES IN COMMERCIAL SOYBEAN LECITHIN

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SUMMARY

   Gradient high-performance liquid chromatography (HPLC) on silica appears to be inadequate for resolving all classes of phospholipids in complex mixtures. We have tested patterns obtained with 18 predefined phospholipids and β-lysophospholipids, demonstrating the relationship between the major-class phospholipids and their β-lyso derivatives. A poor resolution results when many phospholipids are mixed and co-chromatographed. We analysed some of the factors involved, and showed that the insufficient resolution in discrete regions of the HPLC profile of such mixtures is caused by both intrinsic structural properties within each phospholipid class (*e.g.*, heterogeneity in aliphatic residues), which cause severe expansion of the eluted peaks, and by changes related to column performance. A great improvement in resolution is attained by using thin-layer chromatography to resolve the phospholipids in the congested regions of the HPLC pattern. This combination is exemplified on a commercially available, simple mixture, soybean lecithin.

INTRODUCTION

   Phospholipids (PL) are major constituents in biological membranes. In addition to serving as structural units forming the bilayer, they are also active components which turn over in a complex manner that may be crucial for membrane function in general and for transducing signals from the surrounding environment into the cell in particular.
   Only a few studies have appeared in the last decade in attempts to use high-performance liquid chromatography (HPLC) for identifying or separating phospholipid classes[1–7]. Most of these studies have improved some aspects of the technology and have contributed valuable data to the art, yet in some this was accompanied by some drawbacks, *e.g.*, employing acids that may hydrolyse vinyl ether bonds or using phosphoric acid, which complicates phosphorus determination. In this work we used the protocol described by Geurts van Kessel and co-workers[1,2]. This method is based

on unmodified silica as the stationary phase and a ternary mixture of the weakly absorbing solvents *n*-hexane, isopropanol and water as the mobile phase. It is well recognized that the larger the number of phospholipids to be resolved, the greater is the likelihood of overlapping of peaks in the elution profile[3,6]. To overcome this problem, a combination of HPLC and thin-layer chromatography (TLC) is suggested for extensive analyses of phospholipids.

EXPERIMENTAL

Phospholipids

Phosphatidylcholine (PC, type IX-E), L-α-phosphatidylethanolamine (PE, type III), L-α-phosphatidyl-DL-glycerol (PG), L-α-phosphatidic acid (PA), diphosphatidylglycerol (CAR, cardiolipin), all from egg yolk, phosphatidylcholine type IV from soybean (SBL), and the synthetic phospholipids dipalmityolphosphatidylmono- and dimethylethanolamine (PMME and PDME) were all purchased from Sigma (St. Louis, MO, U.S.A.). Phosphatidylinositol (PI, grade I) from egg yolk, phosphatidylserine (PS, grade I) from bovine spinal cord and brain sphingomyelin (Sm) were bought from Lipid Products (South Nutfield, U.K.).

Other chemicals

All chemicals were of analytical-reagent or HPLC grade. Chloroform, methanol and acetic acid were bought from Frutarom (Haifa, Israel), *n*-hexane, isopropanol from Bio-Lab (Jerusalem, Israel), methylamine (40% solution in water) from Fluka (Buchs, Switzerland) and perchloric acid, ascorbic acid and ammonium molybdate from BDH (Poole, U.K.).

[32P]Phospholipids

[32P]-labelled phospholipids were prepared from rat basophilic leukaemia (RBL) cells[8,9] supplemented with [32P]orthophosphoric acid (8.5 μCi/ml) on day 3 for 45 h. The radioactive medium was discarded, cells were washed four times with Tris–saline (pH 7.4) and cellular lipids were extracted as described previously[10,11]. The crude lipid extract was loaded on a silica gel G TLC plate and developed with light petroleum–diethyl ether–acetic acid (80:20:1) to remove neutral lipids. The phospholipid band at the origin was scraped off, extracted three times with chloroform–methanol–water (65:25:4) and spun to remove silica particles before use.

All phospholipids were dissolved in *n*-hexane–isopropanol–water (40:54:6).

Phosphorus determination

Stock solutions or fractions collected after HPLC were analysed for phosphorus by the method of Rouser *et al.*[12], scaled down as follows. Samples in 10 × 13 mm glass test-tubes were dried, supplemented with 140 μl of concentrated (70%) perchloric acid, covered with glass onions, heated at 180°C for 60 min, cooled and briefly spun. An 800-μl volume of the colour reagent (composed of 5 parts of water, 2 parts of 1.25% ammonium molybdate and 1 part of freshly prepared 10% ascorbic acid) was added to each tube and to tubes containing 25 nmol of standard phosphate solution (Sigma 661-9), and the mixture was heated at 100°C for 5 min, then read at 820 nm.

Preparation of lysophospholipids

A 150–400-nmol amount of commercial phospholipids (PA, PG, PMME and PDME) or of [^{32}P]phospholipids separated by HPLC (PE, PI, PS and PC) were dried in duplicate under nitrogen and lyophilized overnight. One tube of each lipid was supplemented with 0.3 ml of bee venom phospholipase A$_2$ [1–3 μg/ml in digestion buffer composed of 15 mM Tris (pH 8.1), 124 mM NaCl, 5 mM KCl, 3 mM CaCl$_2$ and 50 μg of immunoglobulin G (IgG) as a protein carrier], sonicated for 2 min in a bath sonicator (Laboratory Supplies, Hicksville, NY, U.S.A.) and incubated for 5 h at 40 ± 1°C. The second tube in each pair was treated alike but the enzyme was omitted from the buffer. The phospholipase A$_2$ (E.C. 3.1.1.4) (a gift from Dr. Phillip Lazarovici) had a specific activity of 1 mmole of lecithin/μg of enzyme/h at 37°C.

The aqueous mixtures were extracted after digestion as described for the ^{32}P-labelled cells. Extracts within each pair were combined and chromatographed.

HPLC analysis

HPLC analysis was performed with a Waters system (Waters Assoc., Milford, MA, U.S.A.) using two Model 6000 A solvent delivery units and a Model 660 solvent flow programmer. The sample was applied with a Hamilton syringe via a U6K injector and the elution was monitored with an M-480 variable-wavelength UV detector, set at 206 nm.

All separations were performed on a stainless-steel column (250 × 4.6 mm I.D.) home packed with 5 μm LiChrosorb Si-100 silica (Merck, Darmstadt, F.R.G.). Separations were performed at room temperature (*ca.* 20°C) and, unless stated otherwise, a flow-rate of 1 ml/min was maintained. The column was washed with solvent mixture B (*n*-hexane–isopropanol–water, 60:80:14) until a steady baseline was established, followed by mixture A (*n*-hexane–isopropanol–water, 60:80:7) until a new baseline (approximately 0.07 absorbance units above the former) was established. Injected samples varied between 10 and 200 μl, containing 5–30 nmol of individual phospholipids or 0.2–2 μmol of mixtures.

The column was eluted for 5 min with solvent A, followed by a 20 min (5–25 min) linear gradient between 0 and 100% solvent B in A and then by further isocratic elution in solvent B until 45–70 min as required. Using absorbance at 206 nm for detection, a drop in the solvent absorbance baseline was detected only 9.5 min after the gradient had been effected (arrow and dashed line in Fig. 1A).

Calculations. Corrected retention times were given by $t' = t_L - t_0$, where t_L is the actual retention time of a given lipid and t_0 the elution time of unretained compound (*i.e.*, the solvent front), 3.8 min. The resolution (R) between two peaks (A and B) was given by $R = 2 (t_A - t_B)/(w_A + w_B)$, where w is the peak width at the baseline. The peak absorbance was calculated by weighing the peak area cut from a photocopy of the chromatogram.

Thin-layer chromatography

Pre-coated silica gel G glass plates (0.2 mm thick) were used for TLC. Samples were applied as spots 15 mm above the lower rim of the plate and dried with a warm-air fan. Chromatograms were developed in either of two solvent systems[13], a basic system consisting of chloroform–methanol–40% methylamine (39:18:4.5) or an acidic system consisting of chloroform–acetone–methanol–acetic acid–water

(30:12:6:9:3), in a 9 × 14 × 14 cm tank containing 100 ml of the solvent mixture at room temperature. The plates were removed as soon as the solvent front had reached the pre-marked distance of 8 cm, dried, exposed to iodine vapour and the spots circled. Phospholipids were revealed by using a spray cocktail prepared as previously described[14], positive spots becoming blue within a few minutes.

RESULTS

HPLC analysis of standards

In order to set up the HPLC system, we analysed individual, commercially available phospholipids. Fig. 1 shows the elution profile of such standards. Panel A depicts the pattern of a co-chromatographed mixture of phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidic acid (PA) and sphingomyelin (Sm). Panel B contains an overlay of elution profiles of four individual
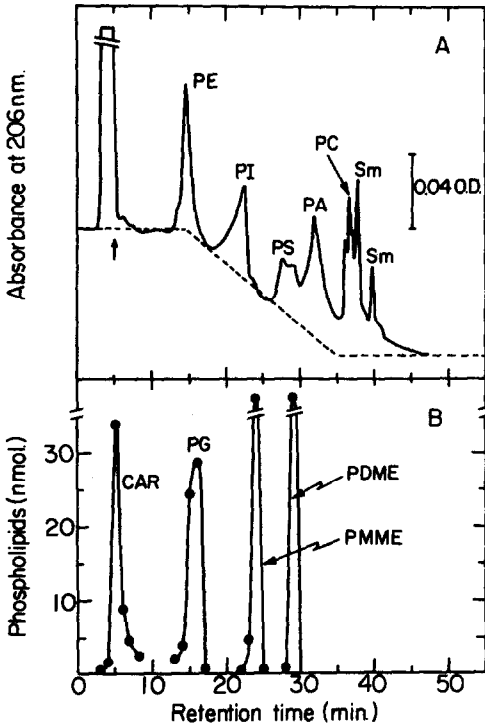

Fig. 1. Retention profile of commercially available phospholipid markers. (A) Absorbance chromatogram obtained for a mixture of the specified phospholipids, injected in 80 μl, eluted for 5 min with *n*-hexane–isopropanol–water (60:80:7), then with a 20 min linear gradient ending with *n*-hexane–isopropanol–water (60:80:14). Column: 250 × 4.6 mm I.D. packed with LiChrosorb Si-100, 5 μm. Flow-rate: 1 ml/min. Phospholipids: PE (11 nmol), PI (35 nmol), PS (35 nmol), PA (39 nmol), PC (35 nmol) and sphingomyelin (90 nmol). The dashed line marks the baselines for the two solvent mixtures and the theoretical shape of the gradient; the arrow marks initiation of the gradient. (B) Phospholipid phosphate elution profiles for four additional phospholipids. The phospholipids (Car, PG, PMME and PDME, 67 nmol of each) were individually injected in 50 μl, run as described in (A), collected in a 1 min fraction, and phospholipids were determined as detailed under Experimental.
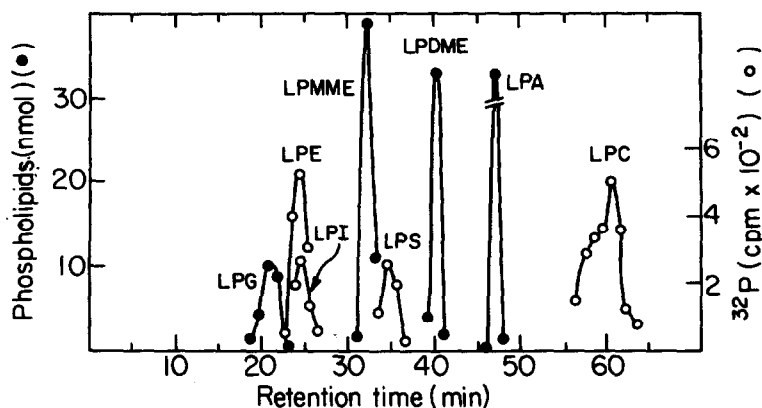
Fig. 2. Retention profile of lysophospholipids. β-Lysophospholipids were prepared as described under Experimental and run under the conditions detailed in Fig. 1. [32]P radioactivity and phosphate chromatograms of the individual lipids were drawn on a common time scale. "L" in each abbreviation represents "lyso" of the respective lipid.

phospholipids: diphosphatidylglycerol (cardiolipin, CAR), phosphatidylglycerol (PG) and the two intermediate products of the phospholipid methylation pathway, phosphatidylmonomethylethanolamine (PMME) and phosphatidyldimethylethanolamine (PDME).

Examination of the relative retention times reveals that all four major phospholipids (PC, PE, PI and PS) elute with good resolution and in the same order as found elsewhere using similar set-ups[1,5,7]. PA, however, elutes with an uncorrected retention of approximately 30 min, between PS and PC, and not between PE and PI[5] or at the leading edge of the solvent front, with the unretained material[1]. Cardiolipin, however, appears in the latter position. In accordance with earlier reports, sphingomyelin elutes as two well resolved peaks[1,3], the faster component of which partially overlaps with PC. The complication that arises on superimposing the two panels of Fig. 1 is notable, namely that PG may occasionally be covered (depending on the relative amounts) by the tail of PE and PDME would usually be masked by PS.

β-Lysophospholipids (LPL)

β-Lyso derivatives of phospholipids are intermediate products in many pathways of phospholipid turnover reactions. We have prepared these derivatives (see Experimental) for assessing their retention patterns, and co-chromatographed each lyso derivative with its own original (undigested) parent phospholipid. This protocol ascertained that no adverse reaction(s) other than the hydrolysis of the fatty acid had taken place (the parent phospholipid had been sham-digested), and only one new product had been formed in the enzymatic digestion with phospholipase A_2, logically referred to as the appropriate β-lysophospholipid. It also rendered more accurate the comparison between the retentions of the substrate and the product. The retention profiles of β-lysophospholipids thus derived from eight individual runs are given in Fig. 2. Apart from LPI and LPE, all of these products are resolved well from each other in this system, and with one exception, LPI (see comments for Table I), each is well resolved from its respective intact phospholipid. As shown in Table I, we

TABLE I

INTERRELATIONS BETWEEN ELUTION PROFILES OF β-LYSOPHOSPHOLIPIDS AND THEIR RESPECTIVE PHOSPHOLIPIDS

Lipid*	Retention time, t (min)		Δt	t'_{LP}/t'_{P}**
	Parent phospholipid	β-Lysophospholipid		
PE	13.3	24.4	11.1	2.2
PG	16.2	21	4.8	1.4
PI***	22.5	24.5	2	1.1
PMME	23.6	32.3	8.7	1.4
PS	27.5	34	6.5	1.3
PDME	29.5	40.3	10.8	1.4
PA	32	47.5	15.5	1.5
PC	36	60.5	24.5	1.8

* Pairs are listed in order of the retention times of the parent lipid. Abbreviations are given under Experimental.

** $t' = t - t_0$; t_0 = solvent front retention time, 3.8 min. t'_P and t'_{LP} are the corrected retentions (t') for the intact phospholipid and its β-lyso derivative, respectively.

*** Only trace amounts were hydrolysed in this digestion system. The values were derived from a shoulder on the tailing slope of the PI peak and may not be as rigorous as the others.

attempted to delineate some common patterns in the behaviour of β-lyso derivatives relative to those of the intact phospholipids. The difference between retention times (Δt) and the ratio between the corrected retention times (t'_{LP}/t'_P) were calculated. All eight β-lyso derivatives were retained longer than their parent compounds (Table I and Figs. 1 and 2), and with some exceptions the longer the retention time of the intact phospholipid the larger was the difference in retention times (Δt). In contrast, the average and standard error of the ratio t'_{LP}/t'_P was 1.51 ± 0.09. Three of the tested pairs yielded values within this range (1.42–1.60); however, six of the eight values fell within 16% off this average (1.27–1.76), the extreme exceptions being PE and PI. Thus a rough approximation can be calculated for additional β-lyso derivatives if the elution pattern of the parent compound is known. In relating this ratio to the molecular properties of the lipid pairs no significant distinction could be made between negatively charged and zwitterionic pairs. β-Lysophospholipids of 1-alkenyl and 1-alkyl phospholipids were not detectable as separate peaks. This could result from insufficient amounts present in the digested substrate or from retention times similar to those of the 1-acyl analogues. The retention times of α-lysophospholipids derived from acid hydrolysis of 1-alkenyl-2-acyl-PE or -PC resemble their respective β-lyso-1-acyl analogues (data not shown).

An attempt to resolve the phospholipids and β-lyso derivatives presented so far in this HPLC set-up showed greater complexity. A few congested regions may be expected at retentions of 20–25, 28–35 and 40 min. In the following sections we examine some of the factors that may contribute to this poor resolution.

Heterogeneity of the phospholipids

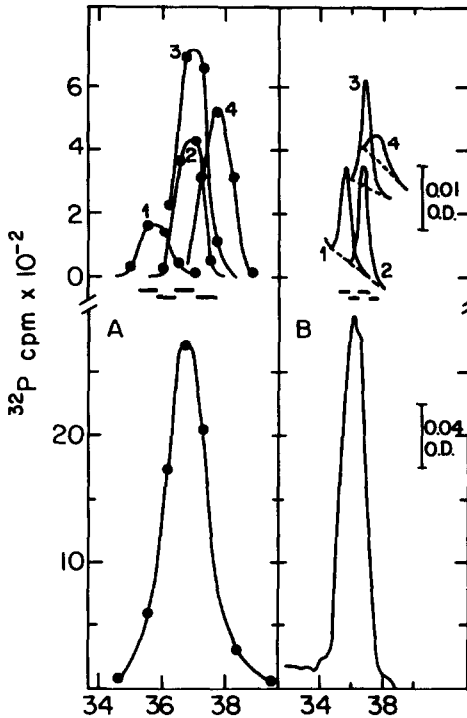The peak width in naturally occurring phospholipids is probably the major

Fig. 3. Structural heterogeneity in the ^{32}P-labelled phosphatidylcholine peak of rat basophilic leukaemia cells phospholipids. The experiment was performed as described in the text. (A) ^{32}P radioactivity; (B) absorbance at 206 nm. The original peak (bottom chromatogram) was subdivided into 4 × 0.5 min fractions (marked by the four horizontal bars), which were re-run individually (top chromatograms). Fractions were numbered by order of retention. See also Table II.

cause of the fair resolutions obtained when a few phospholipid classes are co-chromatographed. This may happen since entire families of species comprise each of the major classes, grouped according to the base in the polar headgroup. Subtle differences in retention attributed to differences among 1,2-diacyl-, 1-akenyl-2-acyl (plasmalogens) and 1-alkyl-2-acylphospholipids have not been studied rigorously; nor have minor differences been attributed to phospho- as opposed to phosphono-lipids

TABLE II

ANALYSIS OF PHOSPHATIDYLCHOLINE PEAK FRACTIONS SHOWN IN FIG. 3

Fraction*	Absorbance (206 nm)	^{32}P radioactivity (cpm)	Normalized absorbance**
1	0.014	374	3.74
2	0.017	907	1.87
3	0.017	1067	1.59
4	0.004	1240	0.32

* Fractions numbered in order of elution (1 elutes first).
** Absorbance at 206 nm divided by cpm (× 10^5).

in any HPLC system. More extensive reference has been made, however, to differences in retention due to the aliphatic residue composition of a given phospholipid group. It has been shown, for instance, that between two phospholipids having two identical *cis*-monoenoic fatty acids, the one with the longer acid runs faster (elutes earlier) on normal-phase silica gel under identical conditions. We have approached this aspect, testing the four major phospholipids PE, PI, PS and PC as follows: The lipids were isolated from ^{32}P-labelled RBL cells (see Experimental), chromatographed as described, and fractions of 0.5 ml (instead of 1 ml) were collected. Aliquots of the fractions containing these four major phospholipids (four fractions in each peak) were individually re-chromatographed and monitored for both absorbance and radioactivity. The results obtained for PC (which were similar to those observed with PE, PS and PI) are shown in Fig. 3. The patterns of the original peak and the re-chromatographed fractions are shown at the bottom and top of the figure, respectively. These clearly show that the individual fractions elute in a comparable manner to their original position, indicating that a genuine structural heterogeneity causes the peak broadness rather than artefacts of overloading or factors related to basic behaviour of liquid chromatography[15]. Further, normalizing the absorbance peak to the radioactive phosphate (Table II) clearly shows a decrease in molar absorbance with the slower eluting species. The absorbance at 206 nm is mainly (though

TABLE III

EFFECT OF ELUTION SCHEME ON THE RETENTION PROFILE

Lipid	Scheme*					
	A		B		C	
	t (min)	R**	t (min)	R**	t (min)	R**
PE	14.4		14.8		20.8	
		2.6		1.6		2.4
PI	22.2		17.9		26.6	
		1.4		1.4		1.7
PS	28		22.4		31.1	
		1.2		0.7		0.82
PA	31.8		25		33.4	
		2.1		2.5		1.86
PC	36.6		31.8		38.6	
		0.75		0.84		0.87
SM$_1$	37.7		33.7		40.4	
		2		1.3		2.3
SM$_2$	39.7		35.7		43.3	

* Scheme A: as under Experimental: 5 min solvent A, linear gradient between 100% mixture A and 100% mixture B for 20 min, followed by mixture B at 1 ml/min. The pattern is given in Fig. 1A. Scheme B: 5 min mixture A, followed by 20 min of a convex gradient (no. 4 setting in the Waters Assoc. M660 gradient control unit) and further elution with mixture B, all at 1 ml/min. Scheme C: 5 min elution with solvent mixture A followed by only 15 min of a linear gradient (no. 6 setting of the control unit), but at 0.7 ml/min.

** $R = 2\Delta t/(w_1 + w_2)$, where Δt is the difference in time between two adjacent peaks and w is the width at the base of the peak.

not solely) determined by the degree of unsaturation; hence it seems that the more unsaturated species elute earlier.

Effect of elution scheme on resolution

Attempts to improve the resolution with the given set of stationary and mobile phases were made by modifying the shape of the gradient or the rate of elution. Table III depicts the retention times and resolution factors for the pattern presented in Fig. 1A and for those obtained with two additional modified elution schemes. Evidently, neither of the modified schemes led to any significant improvement in resolution between neighbouring peaks throughout the chromatogram, particularly in the more congested region around PI.

Stability of column performance

The column was stored routinely in *n*-hexane–isopropanol (60:80) (the same mixture as the elution mixtures but without water), and prior to each session it was washed sequentially with both of the water-containing solvent mixtures. However, retardation of a few minutes in retentions were usually observed for PE and PC, with subsequent recovery of the normal performance. A more serious change was the gradual decrease in retention for the negatively charged phospholipids PG, PI and PA, which shifted during 9 months of intermittent use by up to 5 min (faster). Running a mixture of standards was therefore necessary to assist in positive identification of experimental mixtures. This has become a routine opening of each session in our HPLC studies. Procedures for reconditioning of the column (*e.g.*, with acid as in ref. 3) were not efficient. As reported therein[3], we also found that it restores the originally improved resolution rather than the original retention times. The column ceased to perform shortly after accelerated shifts appeared. A second column made with the same batch of silica retained some of the peaks longer, as expected (compare PE and PC in Fig. 4 with Fig. 1). One notable major difference between the two columns was the order of elution of PG and PE, which was reversed. Attempts to reproduce elution patterns using LiChrosphere Si-100, 10 μm, instead of LiChrosorb Si-100, 5 μm, which differ in both the size and shape of the silica grains, failed completely.

Combining HPLC with TLC

We used two solvent systems for development in one- and two-dimensional TLC[13]. Surprisingly, there was no need for two-dimensional TLC for any of the few groups that co-migrate on HPLC. Table IV details the R_F values of the tested phospholipids in either the basic or acidic solvent system. The lipids congested at 20–25 min (PI, LPS, LPG, LPE and PMME) are separated well in the basic system, as are the overlapping pair at 40 min (the slower species of SM and LPDME). The group congested between 28 and 35 min (PS, PDME, PA, LPMME and LPS) are almost completely resolved in basic solvents, but PA may then overlap the PS + LPS spots and one may consider using acidic solvents in which PA migrates fast and PS/LPS show better resolution.

Analysis of soybean lecithin (SBL)

In order to demonstrate the use of the HPLC–TLC combination we studied the crude lecithin from soybean, "asolectin". this crude material contains only 65%

TABLE IV

R_F VALUES OF SELECTED PHOSPHOLIPIDS (PL) AND LYSOPHOSPHOLIPIDS (LPL) IN ONE-DIMENSIONAL TLC DEVELOPED IN BASIC OR ACIDIC SOLVENT SYSTEMS

See Experimental for solvents.

Lipid	Basic solvents		Acidic solvents	
	PL	LPL	PL	LPL
PA	0.15*	?	0.735	0.29
PC	0.43	0.18	0.34	0.10
PDME	0.76	0.54	0.475	0.195
PMME	0.65	0.40	0.54	0.25
PE	0.615	0.34	0.585	0.27
PG	0.475	0.245	0.58	0.30
PI	0.085	0.035	0.255	0.085
PS	0.16	0.11	0.335	0.115
Card.	0.56		0.731	
Sm.	0.30		0.175	

* Spots spread 3–6 mm, so average R_F values should be considered with a range of ± 0.037 or less.

(w/w) of phospholipids, of which only *ca.* 40% is PC, and *ca.* 20% PE. Fig. 4 shows the HPLC analysis of SBL phospholipids. In addition to the two major peaks [PE (17 min) and PC (40 min)], three minor phospholipids between PE and PC and a mixture of at least three minor ones eluting before PE were clearly detectable. The HPLC fractions were taken for TLC for two purposes: to verify or invalidate identification derived from HPLC retention, and to improve the resolution between lipids that co-migrate in HPLC.
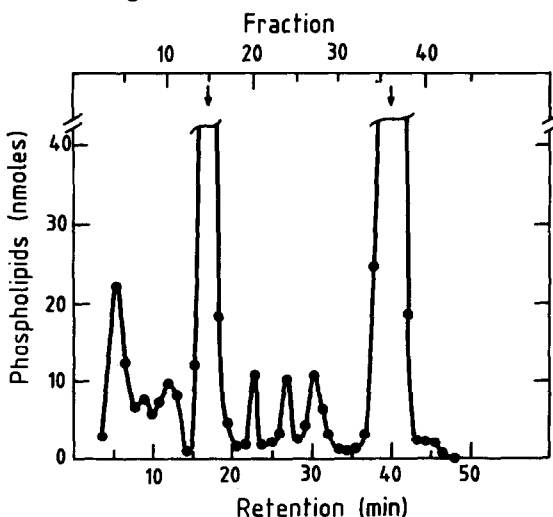

Fig. 4. Soybean lecithin analysis by HPLC. A 2.02 μmol amount of SBL phospholipids was injected in 0.25 ml and chromatographed as described. Fractions (*ca.* 1 ml) were collected and a 0.3 ml sample of each was assayed for phosphate. Note that the fraction numbers (top) differ from the retention times (abscissa). Arrow heads point to the top fractions wherever peaks were trimmed. Recovery of phospholipids was complete.
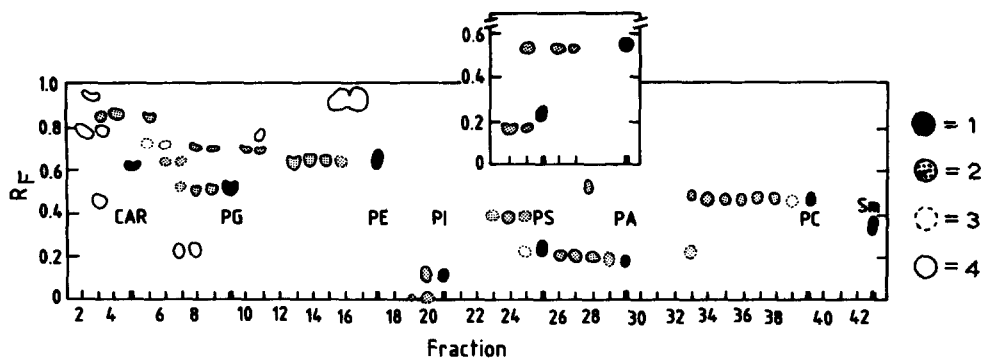
Fig. 5. Thin-layer chromatogram of the HPLC fractions. The remainder of each fraction in Fig. 4, or a volume containing 10 nmol in peak fractions, were concentrated under nitrogen, loaded on silica gel plates and developed as described under Experimental using the basic system of chloroform–methanol–40% methylamine (39:18:4.5). Insert: acidic TLC (see Experimental) for fractions 24–27 for more rigorous identification of the PS/PA region. All spots were stained with iodine vapour. 1, Commercial standards chromatographed individually at positions adjacent to their expected HPLC retention; 2, phosphate-containing spots; 3, very faint iodine-stained spots; negative phosphate in these spots is inconclusive; 4, iodine positive, phosphate negative. Note the variations in R_F values for the standards as compared to Table IV. For abbreviations of standards, see Experimental.

Identification of the various peaks. Fig. 5 shows the TLC pattern of each fraction. Whereas identification of PG (9 min), PE (17 min), PI (23 min), PA (30.5 min) and PC (40 min) on basic (or acidic) TLC confirmed the initial identification according to HPLC retention times, other peaks were shown to be different than suspected. For instance, the peak at 4–6 min (fr. 4) is less polar than cardiolipin (migrating with $R_F = 0.85$ instead of $R_F = 0.62$ of the standard) and no traces of cardiolipin were detectable. The peak at 27 min (fr. 24) may have been suspected to be PS, but the R_F values on both basic and acidic TLC systems indicate a distinctly different entity running faster than PS in the basic system and slower in the acidic system. The relative TLC migrations of lysophosphatidylethanolamine seem very reminiscent of this peak. Sphingomyelin has not been detected by TLC at 45 min, as may have been expected in HPLC.

Improvement of resolution. The multiplicity of neutral lipids and non-phospholipid peaks seen on the plate in Fig. 5 is an outstanding feature of this analysis. This is particularly true for the early fractions, and may potentially cause impurities in the phospholipids with short retentions (*e.g.*, cardiolipin, PG). The PG peak (fr. 7–9) is contaminated by another phospholipid ($R_F = 0.7$) and a non-phospholipid at $R_F = 0.22$. Hence satisfactory analyses to yield pure PG in the HPLC separation may require a preceding step to isolate the phospholipids from their crude source, or a search for different solvent systems. Impurities or overlapping of peaks were also detectable in later fractions: PE (fr. 13–16) overlaps an apolar contaminant with $R_F > 0.9$. PA (fr. 25–29) includes a species with $R_F = 0.52$ (fr. 28) and a species with $R_F = 0.22$ is buried under the leading slope of PC (fr. 33).

DISCUSSION

The complexity of the phospholipid repertoire in nucleated cells is such that it cannot be easily resolved in short (60–70 min) elutions in HPLC. Two main causes

of this difficulty were demonstrated in this study: the heterogeneity in the aliphatic acyl, alkenyl and alkyl species, and the chain length and degree of unsaturation. Highly unsaturated species may elute as fast as 2 min earlier than the saturated homologues (Fig. 3; Table III) and species with longer chains may similarly elute faster relative to those with shorter residues[1]. That the number of acyl species in a given class is related to the peak broadness is also apparent from a brief comparison of the synthetic phospholipids in Fig. 1B (PDME, PMME containing two palmitoyl residues) with the others in Fig. 1A. It appears that the natural phospholipids are sometimes twice as wide as the synthetic ones, consistent with a wider distribution in the more heterogeneous group.

The second factor is the dynamic change in the fine column performance leading to a shift of a few minutes in retention time (see *Stability of column performance*). So long as similar shifts occur with several phospholipid peaks and the order of elution remains unchanged this might not unequivocally be regarded as a deterioration, yet a reduced resolution may be directly effected in some regions of the elution profile, partly owing to condensing the same number of peaks over a shorter period. Not all the changes in retention times relate to "ageing" of the column. For instance, reversion of the order of elution of PG and PE has been observed with two successive columns made from the same batch of silica, in their early days.

Whenever analyses of average properties are desired, especially when the mixture consists of only a few (3–5) classes of phospholipids, separation with this system of HPLC has always proved satisfactory[5,7]. In contrast, many studies of membrane lipid biochemistry deal with intermediate metabolites and other minor species, which thus become the major entity of interest. Such species may be lysophospholipids (when phospholipase A_2 is involved), PA (when headgroup turnover is involved), phosphatidylmono- and -dimethylethanolamine (during phospholipid transmethylation) or phosphatidylmono- and -diphosphoinositol (during numerous cell activation systems). The PI class, for instance, in most cells comprise about 10% of the total cellular phospholipids, of which the mono- and diphosphoinositol phospholipids comprise only 6 and 3%, respectively[16]. Hence these two subclasses, as well as other minor phospholipid classes may comprise 0.1–1% of the total cell lipids and may be readily overrun by the peaks of the major classes. Single phospholipids can be fully recovered in chromatograms of 20 pmole (data not shown). Thus mixtures with as little as 20 nmole (*ca.* 15 μg) total cell phospholipids can be analysed using this HPLC system with no detectable loss of the minor lipids. This study provides a simple combination of HPLC and TLC to resolve the major phospholipids of HPLC fractions.

We have used soybean lecithin, a relatively simple preparation, to exemplify the usefulness of such a combination for the verification of initial identification, and resolution of the classes in congested HPLC traces. If combined HPLC and one-dimensional TLC indicate good resolution for the lipids of interest there should be no further need for the TLC step. The TLC thus serves as a useful tool for an initial assessment of purity. Otherwise this TLC system may serve a good analytical and preparative means because, fortunately, those phospholipids which have poor resolution in the above HPLC system separate well in one of the polar solvent mixtures. Although specific minor lipids may require slight modifications in the solvent systems, the probability that minor phospholipids will be fully resolved from other lipids is much enhanced in the combined system.

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