Effect of FA Chain Length on Normal- and Reversed-Phase HPLC of Phospholipids

Dongmei Zhai and Peter J. Reilly*

Department of Chemical Engineering, Iowa State University, Ames, Iowa 50011

ABSTRACT: Forty-seven saturated synthetic diacyl PA, PC, PE, PG, and PS and five unsaturated diacyl phospholipids (PL) underwent normal- and reversed-phase (RP) HPLC with isocratic isopropanol/hexane/water (5:4:1) and methanol/chloroform/acetonitrile/water (79.5:9:8:3.5) mobile phases, respectively. For normal-phase HPLC, capacity factors (k'_i) decrease with chain length (n) of the two identical PL FA residues, whereas the opposite occurs with RP (C_{18})-HPLC. Plots of ln k'_i vs. n for individual PL classes are in general curved, violating the linear free-energy relationship. For PL of the same n but with different head groups, k'_i with normal-phase HPLC varies as PE < PG < PA < PS < PC, except when $n \geq 16$, when the order is PE < PS ≈ PA ≈ PG < PC. For RP-HPLC, the order of k'_i values is PG < PA ≈ PS < PC ≈ PE until $n \geq 16$, when it is PA ≈ PG < PS << PC ≈ PE. With normal-phase HPLC, k'_i values of PL with unsaturated FA of $n = 18$ are ordered as PE < PA < PC. Increasing degrees of unsaturation lead to increasing k'_i.

Paper no. J10363 in *JAOCS 79*, 1187–1190 (December 2002).

KEY WORDS: Capacity factor, chain length, HPLC, linear free-energy relationship, normal-phase, phospholipid, reversed-phase.

Phospholipids (PL), including PA, PC, PE, PG, and PS, are important in cell structure and signal transduction and are widely used in feeds and foods. PL are valuable because they have both hydrophobic FA chains and hydrophilic phosphate and head groups; thus, they are surfactants and are used as emulsifiers, stabilizers, and wetting agents.

Accurate quantitative analysis of PL is essential in order to understand their roles. HPLC with normal-phase silica or reversed-phase C_8, C_{18}, or octadecylsilyl (ODS) columns is commonly employed to analyze natural PL mixtures, with good separation of lipid classes and molecular species (1–4). Normal-phase HPLC is usually employed to separate different PL classes and those with different head groups, whereas RP-HPLC is normally used to separate different PL of the same class.

An important factor in PL separation is the effect of FA chain length (n) on the retention time of the chromatographic peak. This has not been fully investigated, mainly because most investigators have subjected natural PL mixtures to HPLC (5–7). These cover narrow ranges of n, and most compounds in the mixture are composed of FA residues with different degrees of unsaturation. The same factors were present even when pure synthetic compounds were used (8,9). Two groups (5,7) found that with isocratic elution of reversed-phase columns, ln (retention time) increased linearly with n, but a third (8) did not; it was a major goal of this project to confirm which behavior continued over a wide range of n.

Therefore, to understand further how n affects the HPLC of PL, we have separated eight PA, seventeen PC, eight PE, seven PG, and seven PS, along with five unsaturated PL, with normal-phase silica and reversed-phase C_{18} columns using isocratic elution. In all cases both PL FA residues were of equal n.

EXPERIMENTAL PROCEDURES

Materials. All solvents were from Fisher Scientific (Fair Lawn, NJ) and were HPLC grade. Standards of saturated PA (1,2-diacyl-*sn*-glycero-3-phosphate, monosodium salt), PC (1,2-diacyl-*sn*-glycero-3-phosphocholine), PE (1,2-diacyl-*sn*-glycero-3-phosphoethanolamine), PG {1,2-diacyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)], sodium salt}, and PS (1,2-diacyl-*sn*-glycero-3-phospho-L-serine, sodium salt), where diacyl denotes $[CO(CH_2)_{n-2}CH_3]_2$ and where the standards have the general designation PL *n*:0/*n*:0, were purchased from Sigma (St. Louis, MO) and Avanti Polar Lipids (Alabaster, AL). These two firms also supplied the unsaturated compounds 1,2-dioleyl-*sn*-glycero-3-phosphate (sodium salt) (PA *cis*-18:1/*cis*-18:1), 1,2-dioleyl-*sn*-glycero-3-phosphocholine (PC *cis*-18:1/*cis*-18:1), 1,2-dielaidyl-*sn*-glycero-3-phosphocholine (PC *trans*-18:1/*trans*-18:1), 1,2-dilinoleyl-*sn*-glycero-3-phosphocholine (PC *cis*-18:2/*cis*-18:2), and 1,2-di-oleyl-*sn*-glycero-3-phosphoethanolamine (PE *cis*-18:1/*cis*-18:1). All standards were dissolved in an isopropanol/hexane/water mixture (5:4:1, by vol) to 0.5–1.5 g/L.

Chromatography. Two different HPLC columns were used. One was a Zorbax (Agilent Technologies, Wilmington, DE) SB-C18 column, 250 mm × 4.6 mm i.d., 5-μm particle size, while the other was a 250 mm × 4.6 mm i.d., 5-μm particle size Hichrom (Richard Scientific, Novato, CA) LichroSorb Si60-5 column. The experimental system (10) had an LPM-600 HDU-1 helium degassing unit (Shimadzu Scientific Instruments, Columbia, MD), a Shimadzu LC-600 solvent delivery system, a Rheodyne (Cotati, CA) 7125

*To whom correspondence should be addressed at Dept. of Chemical Engineering, 2114 Sweeney Hall, Iowa State University, Ames, IA 50011-2230. E-mail: reilly@iastate.edu

injector with a 20-µL sample loop, a Shimadzu CR501 Chromatopac integrator, and either a Varex (Burtonsville, MD) IIA ELSD or else a Shimadzu SPD-6AV UV-visible spectrophotometric detector. HPLC was conducted at 22 ± 2°C, and a 0.01–0.03 mg sample was injected into the system. The flow rate of the nebulizing nitrogen in the ELSD was 1.8 mL/min, and the drift tube temperature was 130°C. Nitrogen gas backpressure was around 1.3 bar.

For the reversed-phase column, the mobile phase was an isocratic mixture of methanol, chloroform, acetonitrile, and water (79.5:9:8:3.5, by vol) at 0.52 mL/min. The holdup time t_0 was determined by injecting an aqueous NH_4Cl solution. The mobile phase for the normal-phase column was an isocratic mixture of isopropanol, hexane, and water (5:4:1, by vol) at 0.48 mL/min. Values of t_0 were measured by injecting hexane and measuring the change of absorbance at 204 nm.

Data analysis. The capacity factor k_i is related to the retention time t_{R_i} of component i as

$$k'_i = (t_{R_i} - t_o)/t_o \quad [1]$$

The linear free-energy relationship (11) can be expressed as

$$\ln(k'_i) = (\Delta G_{po} - \Delta G_{io})/RT = \sum_J \tau_{ji} \quad [2]$$

where $\ln k'_i$ is proportional to the free energy change associated with the chromatographic distribution process, p refers to the parent compound, and τ_{ji} is a parameter corresponding to the change in chromatographic retention time upon replacing a hydrogen atom in the parent compound by substituent j (12). This suggests that the appropriate way to plot retention time data is as $\ln k'_i$ vs. n, and that curves representing PL with different head groups should be straight with different intercepts but the same slopes.

RESULTS

RP-HPLC of saturated PL. Separation of the PL by RP-HPLC is shown on Figure 1. Values of $\ln k'_i$ increase with increasing n. The curves for the PC and PE series are nonlinear; their slopes also increase with increasing n. A lack of precision in k'_i values for the PA, PG, and PS series, partially caused by the non-Gaussian nature of their peaks, makes it impossible to discern whether their curves are also nonlinear. At lower n values, k'_i increases in the order PG < PA ≈ PS < PC ≈ PE. At $16 \le n \le 18$, the order is PA ≈ PG < PS << PC ≈ PE.

Normal-phase HPLC of saturated PL. Separation of the five PL classes by normal-phase HPLC is shown in Figure 2. In general the precision of measured values of k'_i is greater than that with RP-HPLC. Values of $\ln k'_i$ decrease with increasing n. In all cases the curves are nonlinear, with their negative slopes decreasing with increasing n. At lower values of n, k'_i increases in the order PE < PG < PA < PS < PC. When $16 \le n \le 18$, the latter value being the highest measured with all PL classes except the PC, the order is PE < PS ≈ PA ≈ PG < PC.

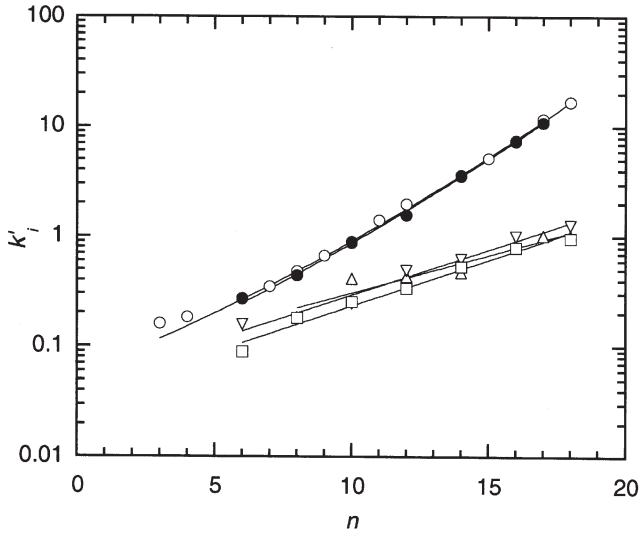

FIG. 1. Effect of n on $\ln k'_i$ of phospholipids (PL) from RP-HPLC. PA: △, PC: ○, PE: ●, phosphatidylglycerol, □, PS: ▽.

Normal-phase HPLC of unsaturated PL. Separation of five unsaturated PL of $n = 18$ from each other and from their corresponding saturated PL is shown in Table 1. As in normal-phase HPLC of saturated PL, values of k'_i increase in the order PE < PA < PC. Increasing degrees of unsaturation lead to increasing values of k'_i. In the one case where it was measured, a PC with a *trans*-double bond eluted slightly before the corresponding PC with a *cis*-double bond.

DISCUSSION

Four different experimental results must be explained, three of them for both normal-phase and RP-HPLC: (i) the effect of n on k'_i values of PL with the same head group; (ii) the effect of FA unsaturation on k'_i values of PL with the same

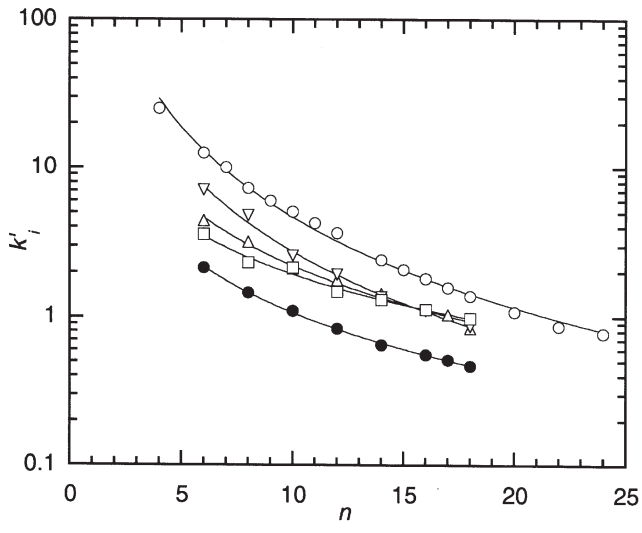

FIG. 2. Effect of n on $\ln k'_i$ of PL from normal-phase HPLC. Abbreviation and symbols as in Figure 1.

TABLE 1
Effect of Phospholipid Unsaturation on Capacity Factors in Normal-Phase HPLC

Phospholipid	18:0/18:0	*cis*-18:1/*cis*-18:1	*cis,cis*-18:2/*cis,cis*-18:2
PA	0.84	1.07	
PC	1.39	1.58, 1.51[a]	1.84
PE	0.47	0.52	

[a]*trans*-18:1/*trans*-18:1.

head group (for normal-phase HPLC only); (iii) the effect of head group on elution order for PL of the same *n*; and (iv) the nonlinearity in most or all of the curves in Figures 1 and 2.

Effect of n *on* k'_i. In RP-HPLC, hydrophobic C_{18} groups on the stationary phase bind to the hydrophobic FA chains of the PL. Therefore, increasing *n*, leading to greater solute hydrophobicity, causes increasing values of k'_i for PL with saturated FA chains (Fig. 1). Two effects are pres-ent with normal-phase HPLC: (i) The hydrophilic silica surface of the stationary phase adsorbs the more hydrophilic end of the PL molecule, encompassing the phosphate and head groups, whose polarities can change with changes in *n*; (ii) the hydrophilic silica surface causes an enrichment of water near it and less chromatographic retardation of more hydrophobic solutes. The second effect especially causes k'_i to decrease with increasing *n* of saturated FA chains (Fig. 2).

Effect of head group on k'_i. Figure 1 shows that values of ln k'_i for RP-HPLC of saturated PA, PG, and PS, of the same *n* are close and are less than those of PC and PE, which are also very close to each other. Lima and Synovec (8) found a PE < PC < PA elution order using a C_{18} stationary phase end-capped with trimethyl groups and eluting with methanol/isopropanol/*p*-toluenesulfonic acid (97.5:2.5:70 mM), very different from our order. Since both stationary and mobile phases vary, it is not possible to isolate the reason for this.

Values of ln k'_i of saturated PL of the same *n* from normal-phase HPLC are found in Figure 2. Notably, PC at all values of *n* elutes last, when it would be expected to elute early if its high hydrophobicity mainly determined elution order. Clearly, elution order is sensitive to both mobile and stationary phases (1–3). For instance, PL elution orders very different from ours occur with silica columns even when only the minor components in an isopropanol/hexane-based mobile phase are changed, while use of acetonitrile-based mobile phases causes further changes (1–3).

Effect of unsaturation on k'_i. Many groups have noted that retention times in RP-HPLC of PL of the same class and value of *n* decrease with an increase of double bonds on FA chains, caused by the decreasing hydrophobicity of the latter. Lin *et al*. (9) further observed that PC having *cis*-FA residues eluted slightly earlier than equivalent *trans*-isomers on a reversed-phase column. Our results with normal-phase HPLC separation of PC of $n = 18$ and increasing degrees of unsaturation, as well as with the separation of PC *cis*-18:1/*cis*-18:1 and PC *trans*-18:1/*trans*-18:1, are the opposite (Table 1), as would be expected.

Nonlinearity of ln k'_i *vs.* n. We found nonlinear relationships between ln k'_i and *n* for at least two of the five PL classes subjected to RP-HPLC and for all five PC classes subjected to normal-phase HPLC (Figs. 1, 2), when linear relationships are expected and are usually found. A number of groups have noted nonlinear relationships between ln k'_i or related measures and alkyl group *n* in homologous series. Examples where this has occurred in RP-HPLC include separation of eight homologous series of ring-substituted alkylbenzoates in C_8 and C_{18} columns eluted by different methanol/water mixtures (13); separation with five different stationary phases from C_1 through C_{18} of eight homologous $C_nH_{2n+1}Z$ series with methanol/water or methanol/acetonitrile mobile phases (14); separation with ODS columns of nitroalkanes and 2-*n*-alkanones with methanol/water and acetonitrile/water mobile phases (15); separation of *n*-alkyl benzoates with Spherisorb ODS-1 columns and THF/water mobile phases, as well as *n*-alkyl benzoates, phenyl- and chloro-*n*-alkanes, *n*-alkane carboxylic acid methyl esters, and 2-*n*-alkanones with C_{14} columns and acetonitrile/water mobile phases (16); and separation with ODS-2 columns of alkyl aryl ketones with THF/water mobile phases (17). Apparently, the only previous challenge to the linearity of ln k'_i vs. *n* plots for PL in RP-HPLC was by Lima and Synovec (8), who found that PA, PC, and PE of $12 \leq n \leq 18$ behaved nonlinearly when separated with a trimethyl end-capped C_{18} stationary phase and a methanol/isopropanol/*para*-toluenesulfonic acid mobile phase. Deviations from linearity can be both positive (increasing slope with increasing *n*) (8,13,15) and negative (14,16,20). Apparently, no data showing nonlinearity previously existed for PL separation by normal-phase HPLC.

Separation of solutes by HPLC is a complex phenomenon influenced by the molecular structure of the solute itself, the properties of the stationary and mobile phases, and the temperature. Separation of PL is especially difficult to understand, as they have regions that are radically different, with polar phosphate and head groups and two nonpolar FA chains linked to the phosphate group through a glycerol moiety. A further complication is the ionic charges on their phosphate and head groups, which are less than they would be in water, which has a dielectric constant (ε) of 80, but are still substantial in the methanol/chloroform/acetonitrile/water mobile phase ($\varepsilon = 30$) used for RP-HPLC and in the isopropanol/hexane/water mobile phase ($\varepsilon = 20$) used for normal-phase HPLC. Although several relatively recent reviews on RP-HPLC (22–24) provide possible reasons for nonlinearity of plots of ln k'_i vs. *n* for simpler solutes, none is applicable for solutes with such complex structures as the PL, except to indicate that such behavior is quite possible.

In this article we have demonstrated the following: (i) Both normal-phase and RP-HPLC discriminate between PL of different values of *n*, even though the former is not commonly used to separate them; (ii) as previously noted, normal-phase HPLC performs better in separating PL of different classes; (iii) PC, and almost surely other PL of varying degrees of unsaturation are separated by normal-phase HPLC in an order opposite that by RP-HPLC, in both cases as a

function of solute hydrophobicity; (iv) separation of most and perhaps all PL series by normal-phase and RP-HPLC does not obey the linear free-energy relationship, an observation that is understandable given the complexity of the PL molecular structure.

ACKNOWLEDGMENTS

This work was supported by a grant from the U.S. Department of Agriculture through the Biotechnology Byproducts Consortium, a grouping of Iowa State University, the University of Iowa, and the City of Cedar Rapids, Iowa. The authors gratefully thank Professors Tong Wang and Earl Hammond of the Department of Food Science and Human Nutrition at Iowa State University for the use of their high-performance liquid chromatograph and ELSD.

REFERENCES

1. Laganiere, S., Overview of Analytical Methods for Phospholipid Studies, in *Phospholipids—Biochemical, Pharmaceutical and Analytical Considerations*, edited by I. Hanin and G. Pepeu, Plenum, New York, 1990, pp. 13–41.
2. Abidi, S.L., and T.L. Mounts, High-Performance Liquid Chromatography of Phosphatidic Acid, *J. Chromatogr. B 671*:281–297 (1995).
3. Christie, W.W., Separation of Phospholipid Classes by High-Performance Liquid Chromatography, in *Advances in Lipid Methodology—Three*, edited by W.W. Christie, The Oily Press, Dundee, Scotland, 1996, pp. 77–107.
4. Bell, M.V., Separations of Molecular Species of Phospholipids by High-Performance Liquid Chromatography, in *Advances in Lipid Methodology—Four*, edited by W.W. Christie, The Oily Press, Dundee, Scotland, 1997, pp. 45–82.
5. Smith, M., and F.B. Jungalwala, Reversed-Phase High-Performance Liquid Chromatography of Phosphatidylcholine: A Simple Method for Determining Relative Hydrophobic Interaction of Various Molecular Species, *J. Lipid Res. 22*:697–704 (1981).
6. 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. 23*:190–196 (1982).
7. Brouwers, J.F.H.M., E.A.A.M. Vernooij, A.G.M. Tielens, and L.M.G. van Golde, Rapid Separation and Identification of Phosphatidylethanolamine Molecular Species, *J. Lipid Res. 40*:164–169 (1999).
8. Lima, L.R., III, and R.E. Synovec, Isocratic Mixed Mode Liquid Chromatographic Separation of Phospholipids with Octadecylsilane-Silica Stationary Phases, *Talanta 41*:581–588 (1994).
9. Lin, J.-T., T.A. McKeon, C.L. Woodruff, and J.A. Singleton, Separation of Synthetic Phosphatidylcholine Molecular Species by High-Performance Liquid Chromatography on a C_8 Column, *J. Chromatogr. A* 824:169–174 (1998).
10. Wang, T., E.G. Hammond, J.L. Cornette, and W.R. Fehr, Fractionation of Soybean Phospholipids by High-Performance Liquid Chromatography with an Evaporative Light-Scattering Detector, *J. Am. Oil Chem. Soc. 76*:1313–1321 (1999).
11. Martin, A.J.P., Some Theoretical Aspects of Partition Chromatography, *Biochemical Society Symposium No. 3, Partition Chromatography*, edited by R.T. Williams and R.L.M. Synge, Cambridge University Press, Cambridge, 1950.
12. Chen, B.-K., and C. Horváth, Evaluation of Substituent Contributions to Chromatographic Retention: Quantitative Structure–Retention Relationships, *J. Chromatogr. 171*:15–28 (1979).
13. Tomlinson, E., H. Poppe, and J.C. Kraak, Thermodynamics of Functional Groups in Reversed-Phase High-Performance Liquid–Solid Chromatography, *Intl. J. Pharmaceut. 7*:225–243 (1981).
14. Tchapla, A., H. Colin, and G. Guiochon, Linearity of Homologous Series Retention Plots in Reversed-Phase Liquid Chromatography, *Anal. Chem. 56*:621–625 (1984).
15. Smith, R.M., and N. Finn, Comparison of Retention Index Scales Based on Alkyl Aryl Ketones, Alkane-2-ones and 1-Nitroalkanes for Polar Drugs on Reversed-Phase High-Performance Liquid Chromatography, *J. Chromatogr. 537*:51–60 (1991).
16. Heron, S., and A. Tchapla, Description of Retention Mechanism by Solvophobic Theory: Influence of Organic Modifiers on the Retention Behaviour of Homologous Series in Reversed-Phase Liquid Chromatography, *J. Chromatogr. 556*:219–234 (1991).
17. Smith, R.M., and R. Wang, Retention Prediction of Analytes in Reversed-Phase High-Performance Liquid Chromatography Based on Molecular Structure. VII. Separations Using Tetrahydrofuran-Buffer Eluents, *J. Chromatogr. 558*:7–18 (1991).
18. Tchapla, A., S. Héron, E. Lesellier, and H. Colin, General View of Molecular Interaction Mechanisms in Reversed-Phase Liquid Chromatography, *J. Chromatogr. A 656*:81–112 (1993).
19. Smith, R.M., Functional Group Contributions to the Retention of Analytes in Reversed-Phase High-Performance Liquid Chromatography, *J. Chromatogr. A 656*:381–415 (1993).
20. Smith, R.M., Retention Prediction Based on Molecular Structure, *Journal of Chromatography Library, Vol. 57: Retention and Selectivity in Liquid Chromatography*, edited by R.M. Smith, Elsevier, Amsterdam, 1995, pp. 1–46.

[Received June 24, 2002; accepted August 13, 2002]