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Gradient elution reversed-phase chromatographic isolation of individual glycerophospholipid molecular species

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Abstract

We describe a gradient elution reversed-phase high-performance liquid chromatographic approach for isolation of individual glycerophospholipid molecular species which greatly improves resolution and reduces run time compared to isocratic techniques. Separations were optimized and elution order and retention time data established by synthesizing 37 different homogeneous phospholipids comprising the major alkylacyl, diacyl and plasmalogen molecular species in samples derived from mammalian sources. Empirical equations which predict the elution order of individual species were derived. The method was validated with the use of complex mixtures of choline and ethanolamine glycerophospholipid species from isolated rabbit cardiomyocytes and porcine endothelial cells. © 1997 Elsevier Science B.V.

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1. Introduction

The tremendous structural diversity among glycerophospholipid molecules mandates the use of high resolution chromatographic techniques to provide a comprehensive characterization of phospholipid composition in samples derived from biologic sources. This is generally achieved by a multi-step approach based on recovery of glycerophospholipids by organic solvent extraction, separation into classes based on differences in polar headgroup composition at the *sn*-3 position and finally, resolution of individual molecular species within a class based on differences in the composition of the aliphatic groups at the *sn*-1 and/or *sn*-2 positions of the glycerophospholipid molecule [1–3]. To isolate the individual species intact (i.e. without derivatization), the traditional approach is to utilize isocratic reversed-phase HPLC with a stationary phase comprised of fully 'end-capped' octadecylsilyl (C_{18})-derivatized 3-5 μm microparticles and a mobile phase comprised of acetonitrile–methanol–water containing a cationic amine salt (e.g. choline chloride) [4]. This isocratic approach may require elution of the column for four or more hours [5]. Even with extended elution times, it has not been possible to resolve all of the individual phospholipid species present in complex biologic mixtures.

After separation of the major components in the sample, it is necessary to identify the phospholipid species present in each individual peak. This is generally accomplished by structural analysis of the

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components present in the isolated peaks utilizing gas chromatography (GC) or GC–mass spectrometry (GC–MS) after derivatization to form volatile derivatives or by using fast-atom bombardment MS (FAB-MS) techniques applied to the intact phospholipid species. The identification of all individual species in the chromatogram is often difficult due to: (1) incomplete resolution of all species following HPLC resulting in the presence of multiple species coeluting in the same peak or of a single species eluting in multiple adjacent peaks (2) the presence of very small quantities of some components (3) ambiguities arising from the presence of multiple volatile derivatives which are not completely resolved by GC, (4) the presence of isobaric species which cannot be readily distinguished by MS approaches and (5) the introduction of contaminating lipids during the course of analysis including derivative preparation (if required). Accordingly, some of the published information regarding elution order and retention time data for some phospholipid molecular species may be in error. Furthermore, detailed retention data have been provided almost exclusively for diacyl phospholipid species with limited information available for alkylacyl and plasmalogen (alkenylacyl) molecular species.

The present study was undertaken to identify chromatographic conditions which would reduce the time required to isolate the major phospholipid molecular species while improving resolution at the same time by employing a gradient elution approach. To avoid the ambiguities arising from traditional approaches to peak identification and to provide retention time data for alkylacyl and alkenylacyl (plasmalogen) phospholipids in addition to diacyl phospholipids, we synthesized 37 different individual homogeneous diradyl choline glycerophospholipid molecular species corresponding to those most likely to be encountered in tissue samples from mammalian species. The retention characteristics of each compound following reversed-phase HPLC chromatography were then determined and a quantitative relationship between molecular structure and retention time established. To verify the utility of the gradient-elution approach, we compared the resolution of individual molecular species of rabbit cardiomyocyte and porcine endothelial cell choline and ethanolamine glycerophospholipids by isocratic and gradient elution approaches. Finally, we demonstrated an excellent correlation between peak assignments determined from retention time data of synthetic phospholipids with assignments made based on peak identification using GC and FAB-MS techniques.

2. Experimental

2.1. Characterization of UV absorption spectra

The absorption spectra of isolated synthetic homogeneous phospholipid molecular species were characterized using a Beckman DU-70 spectrophotometer and quartz cuvettes. Phospholipid samples were dissolved in the mobile phase used for reversed-phase HPLC separation (see below) and the absorbance determined in the 190–340 nm wavelength range against a reference blank comprised of mobile phase only.

2.2. Extraction and isolation of phospholipid classes from rabbit cardiomyocytes

Rabbit cardiomyocytes were isolated following Langendorff perfusion with Tyrode's solution containing collagenase as previously described [6]. Cardiomyoctye phospholipids were then recovered following Bligh and Dyer extraction with chloroform, methanol and water [7]. The extracted phospholipids were then separated into classes by gradient elution chromatography using a mobile phase comprised of hexane–isopropanol–water and a stationary phase consisting of a 5-μm particle size, 250×4.6 mm Ultrasphere-Si column (Beckman) (see Ref. [8] for a complete description of the gradient).

2.3. Isolation of individual phospholipid molecular species by gradient-elution reversed-phase HPLC

Individual phospholipid molecular species were isolated by gradient elution. Sample phospholipids were eluted for 30 min with a mobile phase comprised of methanol–water–acetonitrile (87:6:7, v/v/v) containing 20 m*M* choline chloride (solvent A) followed by a linear increase over 60 min to a mobile phase comprised of methanol–water–acetoni-

trile (76:4:20, v/v/v) containing 20 m*M* choline chloride (solvent B). Solvent composition was held constant at 100% solvent B for an additional 30 min after which the solvent composition was returned to 100% solvent A in a linear fashion over 10 min. With this approach, all phospholipid molecular species eluted within 100–110 min after injection. The stationary phase consisted of a 250×4.6 mm, 5 μm Ultrasphere-ODS (C_{18}) column (Beckman) or a 250×4.6 mm, 5 μm Prodigy ODS(3) column (Phenomenex) with a C_{18} cartridge precolumn (Alltech). We were able to obtain better resolution with the Prodigy column, however, the trade-off for the Prodigy column was that the back pressure was significantly higher and the column life was shorter than that of the Ultrasphere ODS column. The HPLC system was comprised of two Waters 501 pumps, Rheodyne injector, Waters 486 tunable UV absorbance detector, Waters system interface module, NEC PowerMate 386SX computer and Waters baseline chromatography software.

2.4. Structural analysis of glycerophospholipid molecular species in reversed-phase HPLC column effluents

Column effluents corresponding to individual peaks in the HPLC chromatogram were subjected to chloroform extraction to recover sample phospholipids in a 'salt-free' form [8]. Volatile fatty acid methyl ester (FAME) and dimethylacetal (DMA) derivatives were prepared from isolated molecular species containing O-acyl ester-linked (FAME) and 1-O-alk-1′-enyl ether-linked (DMA) aliphatic groups by acid-catalyzed methanolysis [8]. Individual FAME and DMA species were then identified by GC analysis by comparison of retention times with commercially available FAME species (Alltech) or with DMA species prepared from synthetic lysoplasmenylcholine as described [8]. Identification of individual molecular species of choline and ethanolamine glycerophospholipids derived from rabbit cardiomyocytes was based on the GC identification of stoichiometric amounts of FAME an DMA species derived from the *sn*-1 and *sn*-2 aliphatic groups of diacyl and plasmalogen phospholipids and the single FAME species derived from the *sn*-2 group of alkylacyl phospholipids. In selected cases, further structural confirmation was provided by FAB MS analysis of the isolated phospholipid classes and from isolated molecular species as described previously [8].

2.5. Synthesis of individual phospholipid molecular species

For all phospholipid syntheses, the general approach was based on the acylation of the corresponding lysophospholipid species with fatty acid anhydride prepared from commercially available fatty acids (Sigma). All polyunsaturated fatty acids were of the (n-6) series except docosahexaenoic acid (22:6) which was of the (n-3) series. For preparation of alkenylacyl choline glycerophospholipid species (i.e. plasmenylcholine species abbreviated PlasCho), lysoplasmenylcholine (1-O-alk-1′-enyl-*sn*-glycero-3-phosphocholine) was prepared by alkaline hydrolysis of bovine heart choline glycerophospholipid as described previously [9,10]. The product was isolated from the reaction mixture by silicic acid column chromatography using stepwise gradient elution [9]. Characterization of the individual molecular species in the lysoplasmenylcholine product was achieved by GC and FAB-MS [10]. Homogeneous 1-O-hexadec-1′-enyl-*sn*-glycero-3-phosphocholine (16:0 lysoplasmenylcholine), 1-O- octadec-1′-enyl-*sn*-glycero-3-phosphocholine (18:0 lysoplasmenylcholine) and 1-O-octadec-1′,9′- dienyl-*sn*-glycero-3-phosphocholine (18:1 lysoplasmenylcholine) were isolated after the individual lysoplasmenylcholine molecular species were resolved by reversed-phase HPLC [10]. Individual PlasCho (1-0-alk-1′-enyl-2-O-acyl-*sn*-glycero-3-phosphocholine) molecular species were prepared by incubating the appropriate lysoplasmenylcholine species with the corresponding fatty anhydride which was prepared by the dicyclohexylcarbodiimide-mediated condensation of the fatty acid [11]. Lysoplasmenylcholine was acylated at the *sn*-2 hydroxyl group with fatty anhydride utilizing N,N-dimethyl-4-aminopyridine as a catalyst and the PlasCho product was purified by passing the reaction mixture through an amine solid phase extraction column followed by normal-phase HPLC using a Partisil SCX column [12,13]. Synthesis and purification of homogeneous diacyl phosphatidylcholine (1-

O-acyl-2-O-acyl-*sn*-glycero-3-phosphocholine, PtdCho) and alkylacyl choline glycerophospholipid (1-O-alkyl-2-O-acyl-*sn*-glycero-3-phosphocholine, AlkCho) molecular species was performed similarly utilizing the appropriate fatty acid and palmitoyl-, stearoyl-, oleoyl- or linoleoyl-lysophosphatidylcholine (16:0, 18:0, 18:1 or 18:2 lysophosphatidylcholine, respectively, from Avanti Polar Lipids or Sigma) or 1-O-hexadecyl- *sn*-glycero-3-phosphocholine (16:0 lyso-platelet activating factor from Sigma). The final purity of all synthetic products was confirmed by demonstration of comigration of synthetic product and commercially available standards (Avanti Polar Lipids) using two different TLC systems, normal-phase HPLC and following reversed-phase HPLC separation of the individual phospholipid molecular species [4,8,12,13]. The aliphatic chain composition of each PlasCho species was confirmed by the demonstration of stoichiometric quantities of the DMA (corresponding to the *sn*-1 aliphatic group) and FAME (corresponding to the *sn*-2 aliphatic group) derivatives produced following acid catalyzed methanolysis and capillary GC analysis of the PlasCho species [8]. Following acid-catalyzed methanolysis and GC analysis, the composition of PtdCho species was confirmed by the demonstration of stoichiometric production of FAME derivatives corresponding to the *sn*-1 and *sn*-2 aliphatic groups and for AlkCho species, the production of a single FAME derivative corresponding to the *sn*-2 aliphatic group. The concentration of each synthetic phospholipid molecular species was determined by assay of lipid phosphorus and the synthetic products were stored in the dark at −20°C in chloroform–methanol (1:1, v/v) sealed under N_2 in a tapered glass vial with a teflon 'stop–go' cap (Supelco).

3. Results

3.1. UV absorption spectra

The UV absorption spectra of synthetic diacyl, alkylacyl and alkenylacyl choline glycerophospholipid molecular species demonstrate a single absorbance maximum at 203 nm. There was no significant absorption at wavelengths >290 nm. The absorption of UV energy by phospholipids in the 190–210 nm wavelength range reflects the π–π^* electronic transition arising in molecules which contain non-conjugated RCH=CHR′ [14]. Plasmalogen molecular species exhibit proportionately higher molar absorptivity due to the additional absorbance contribution of the vinyl ether (RO–CH=CHR′) group at the *sn*-1 position. For our reversed-phase chromatography, we selected to monitor UV absorbance at the peak absorption wavelength of 203 nm.

3.2. Retention characteristics of individual synthetic phospholipid molecular species

Table 1 compares the retention times for individual phospholipid molecular species. Examination of the retention time data reveals several significant and consistent trends:

(1) For molecular species containing the same aliphatic composition at the *sn*-1 and *sn*-2 positions, retention times increase in the following order: diacyl phospholipids<plasmalogen phospholipids< alkylacyl phospholipids. (Note that when referring to plasmalogen molecular species, all molecular species contain a vinyl ether (–O–CH=CH–) group at the *sn*-1 position even though the shorthand notation given for the *sn*-1 group does not indicate the presence of this additional double bond. Thus, a plasmalogen species designated (16:0,18:1)PlasCho possesses two double bonds, one at the *sn*-2 position and the vinyl ether at the *sn*-1 position. The shorthand notation used to identify individual phospholipid molecular species is described in detail in the footnote of Table 1).

(2) For a series of molecular species containing the same aliphatic group at the *sn*-1 position, retention times increase in the following order: $(R_1, 18{:}3) < (R_1, 22{:}6) < (R_1, 20{:}4) < (R_1, 18{:}2) \ll (R_1, 18{:}1)$.

(3) For a series of molecular species containing the same aliphatic group at the *sn*-2 position, retention times increase in the following order: $(18{:}2,R_2) \ll (16{:}0,R_2) < (18{:}1,R_2) \ll (18{:}0,R_2)$.

In Table 1, relative retention time (RRT) data were also calculated based on the ratio of absolute retention times (ART) of each species to the ART of

Table 1
Retention characteristics for diradyl choline glycerophospholipid molecular species

Composition[a]	ART[b]	RRT[c]	Effective total chain length[d]	Composition	ART	RRT	Effective total chain length
	Diacyl phospholipid (PtdCho) molecular species						
(16:0,18:1)	53.3±1.4	1.306	32.8	(18:0,18:1)	84.0±2.1	2.059	34.8
(16:0,18:2)	40.8±0.5	1.000	31.7	(18:0,18:2)	63.5±0.9	1.556	33.7
(16:0,18:3)	29.2±0.3	0.716	30.5	(18:0,18:3)	49.0±0.5	1.201	32.5
(16:0,20:4)	37.8±0.4	0.926	31.4	(18:0,20:4)	59.9±0.7	1.468	33.4
(16:0,22:6)	33.8±0.9	0.828	31.0	(18:0,22:6)	56.0±0.7	1.373	33.0
(18:1,18:1)	57.6±0.9	1.412	33.2	(18:2,18:1)	41.0±0.6	1.005	31.6
(18:1,18:2)	42.2±0.5	1.034	32.1	(18:2,18:2)	28.9±0.5	0.708	30.5
(18:1,18:3)	31.1±0.8	0.762	30.9	(18:2,18:3)	21.6±0.4	0.529	29.3
(18:1,20:4)	40.3±0.5	0.988	31.8	(18:2,20:4)	27.0±0.5	0.662	30.2
(18:1,22:6)	36.5±0.4	0.895	31.4	(18:2,22:6)	25.2±0.4	0.618	29.8
	Plasmalogen phospholipid (PlasCho) molecular species						
(16:0,18:1)	64.9±1.1	1.591	33.7	(18:0,18:1)	101±2.1	2.475	35.7
(16:0,18:2)	49.6±0.7	1.216	32.6	(18:0,18:2)	78.3±1.2	1.919	34.6
(16:0,18:3)	37.5±0.7	0.919	31.3	(18:0,18:3)	61.0±0.7	1.495	33.4
(16:0,20:4)	46.0±0.8	1.127	32.3	(18:0,20:4)	73.0±0.8	1.789	34.3
(16:0,22:6)	42.6±0.5	1.044	31.9	(18:0,22:6)	66.0±0.8	1.618	33.9
(18:1,18:2)	55.4±0.8	1.358	33.0				
(18:1,20:4)	50.3±0.7	1.233	32.7				
	Alkylacyl phospholipid (AlkCho) molecular species						
(16:0,18:1)	74.8±1.1	1.833	34.4				
(16:0,18:2)	57.3±0.9	1.404	33.3				
(16:0,18:3)	41.3±0.6	1.012	32.1				
(16:0,20:4)	51.0±0.8	1.250	33.0				
(16:0,22:6)	48.4±0.8	1.186	32.6				

[a] The composition of individual phospholipid molecular species is described by the shorthand notation (a:b,c:d) where a and c represent chain length and b and d represent the number of carbon–carbon (C=C) double bonds for the aliphatic groups at the *sn*-1 and *sn*-2 positions, respectively. For plasmalogen molecular species, the double bond present in the vinyl ether (–O–CH=CH–) linkage at the *sn*-1 position is not included in the shorthand notation for these species. All *sn*-2 polyunsaturated fatty acids were of the (n–6) series except 22:6 which was of the (n–3) series.
[b] ART = absolute retention time in minutes (mean±S.D. for 5–8 separate injections).
[c] RRT = relative retention time. The RRT for species X is defined as the ratio of the ART of species X to the ART of (16:0,18:2)PtdCho.
[d] Effective total chain length (ECL_T) was calculated as described in the text.

the (16:0,18:2)PtdCho species. We selected this compound as the reference species because it is commercially available (Avanti Polar Lipids) and because this species is relatively abundant in both choline and ethanolamine phospholipids derived from mammalian sources.

We were able to achieve baseline resolution for most synthetic phospholipid molecular species, however, the following groups of molecular species could not be well resolved by our system: Group 1: (16:0,18:2)PtdCho, (18:2,18:1)PtdCho, (18:1,20:4) PtdCho and (16:0,18:3)AlkCho; Group 2: (16:0,18:2) PlasCho, (18:1,20:4)PlasCho and (18:0,18:3)PtdCho; Group 3: (16:0,18:3)PtdCho and (18:2,18:2)PtdCho; Group 4: (18:0,22:6)PtdCho and (18:1,18:2)PlasCho; Group 5: (18:1,18:1)PtdCho and (16:0,18:2)AlkCho and Group 6: (18:1,18:2)PtdCho and (16:0,22:6) PlasCho.

For isocratic elution of individual diacyl glycerophospholipid molecular species, Patton et al. [4] demonstrated that there was a consistent linear relationship between the logarithm of the ART (or RRT) and the effective carbon number (i.e. effective chain length) of the aliphatic group at the *sn*-1 and *sn*-2 positions. In our gradient elution approach, we found an identical order of elution for diacyl

glycerophospholipid molecular species, however, the relationship between the logarithm of the ART (or RRT) and the effective chain length at the *sn*-1 and *sn*-2 positions was more complex. Accordingly, we derived the following empirical equation to define the effective chain length (ECL) of the R1 and R2 aliphatic groups of a diradyl choline glycerophospholipid molecule:

$$\text{ECL(R1)} = (\text{number carbon atoms at } sn\text{-1 position}) - 1.60\,(\text{number } sn\text{-1 C=C}) + \beta$$

where ECL(R1) = effective chain length of the *sn*-1 aliphatic group, C=C represents a carbon–carbon double bond and the constant β is defined as follows: $\beta = 0$ for diacyl phospholipids, $\beta = 0.9$ for alkenylacyl (plasmalogen) phospholipids and $\beta = 1.6$ for alkylacyl phospholipids. (Note: for plasmalogens, the retention contribution of the vinyl ether (–O–CH=CH–) double bond is included in the β term, this double bond is not included in the determination of the (number *sn*-1 C=C) term).

$$\text{ECL(R2)} = (\text{number carbon atoms at } sn\text{-2 position}) - 1.16\,(\text{number } sn\text{-2 C=C})$$

where ECL(R2) = effective chain length of the *sn*-2 aliphatic group. For the *sn*-2 aliphatic group, we found that the introduction of a C=C group did not increase the retention time to the same extent as for a C=C group at the *sn*-1 position. Note also that all *sn*-2 polyunsaturated fatty acids were of the (n–6) series except 22:6 which was of the (n–3) series. We defined the total effective chain length (ECL_T) as follows:

$$ECL_T = \text{ECL(R1)} + \text{ECL(R2)}$$

Thus, for example, the ECL_T for the (18:1,20:4)PlasCho species is calculated as follows:

$$ECL_T = 18 - (1.60 \times 1) + 0.9 + 20 - (1.16 \times 4) = 32.7$$

The calculated ECL_T for each synthetic phospholipid molecular species is given in Table 1. Comparison of ECL_T values with the predicted order of elution based on ART or RRT values demonstrates that the calculated ECL_T values correctly predict the order of elution (i.e. increasing ECL_T values associated with increasing ART or RRT).

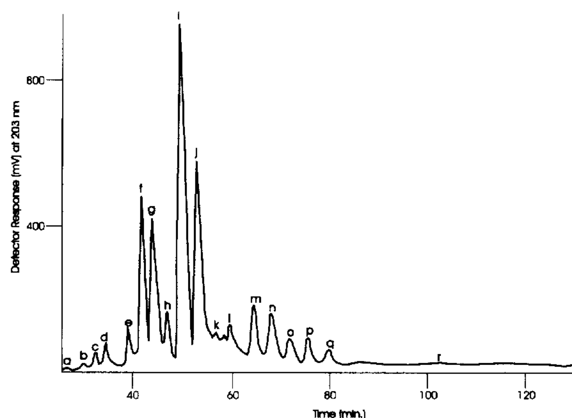

Fig. 1. Reversed-phase HPLC chromatography of individual diradyl choline glycerophospholipid molecular species from isolated rabbit cardiomyocytes. Diradyl choline glycerophospholipid species were separated by gradient elution as described in the experimental section. The small case letters above each peak identify individual molecular species as given in Table 2.

Figs. 1–4 (and data summarized in Tables 2 and 3) demonstrate the utility of our reversed-phase approach to the separation of individual phospholipid molecular species present in rabbit cardiac myocytes and porcine endothelial cells. (Note: the shorthand

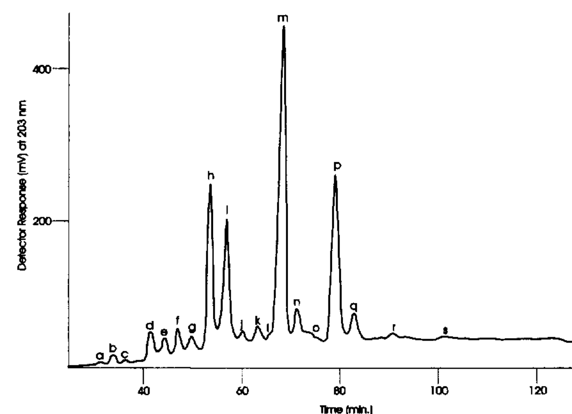

Fig. 2. Reversed-phase HPLC chromatography of individual diradyl ethanolamine glycerophospholipid molecular species from isolated rabbit cardiomyocytes. Diradyl ethanolamine glycerophospholipid species were separated by gradient elution as described in the experimental section. The small case letters above each peak identify individual molecular species as given in Table 2.

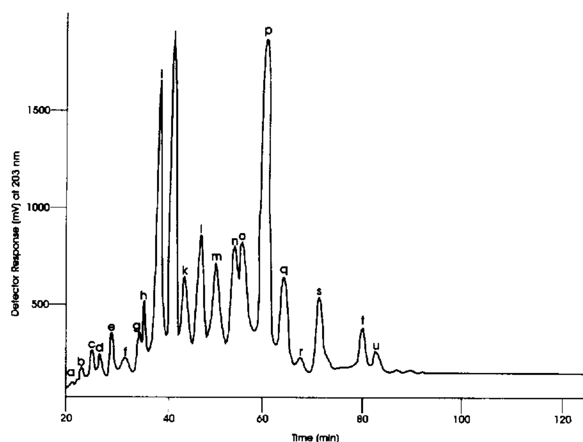

Fig. 3. Reversed-phase HPLC chromatography of individual diradyl choline glycerophospholipid molecular species from porcine endothelial cells. Diradyl choline glycerophospholipid species were separated by gradient elution as described in the experimental section. The small case letters above each peak identify individual molecular species as given in Table 3.

notation used to identify ethanolamine (Eth) glycerophospholipids is identical to that used to identify choline (Cho) glycerophospholipids). When we compared the gradient elution chromatograms shown in Figs. 1–4 with those obtained by the isocratic elution method of Patton et al. [4], we found that gradient elution results in a significant

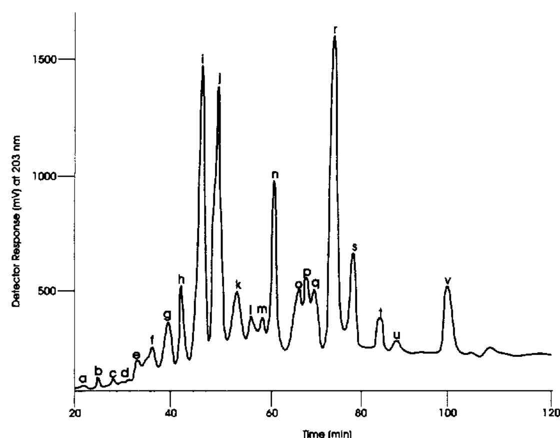

Fig. 4. Reversed-phase HPLC chromatography of individual diradyl ethanolamine glycerophospholipid molecular species from porcine endothelial cells. Diradyl ethanolamine glycerophospholipid species were separated by gradient elution as described in the experimental section. The small case letters above each peak identify individual molecular species as given in Table 3.

increase in the resolution of individual phospholipids and greatly reduces the total time required for elution of all molecular species. The results of the structural characterization of the phospholipid species present in each peak (achieved by GC analysis of volatile FAME and DMA derivatives prepared by acid-catalyzed methanolysis and, in selected cases, by FAB MS analysis of the intact molecular species) are summarized in Tables 2 and 3. The assignment of plasmalogen molecular species was also confirmed based on the lability of these species to acid-catalyzed hydrolysis. Specifically, the choline and ethanolamine plasmalogen peaks 'disappeared' following acid treatment of the choline and ethanolamine glycerophospholipid fractions as previously described [8]. Examination of the data in Figs. 1–4 and Tables 2 and 3 demonstrate that most species elute as single components in separate peaks. For some peaks, multiple species could be identified which coelute. The most significant coelution problem was seen with the (16:0,18:2)PlasCho and (18:1,20:4)PlasCho and corresponding (16:0,18:2) PlasEth and (18:1,20:4)PlasEth species. Although these species coeluted, the mass contribution of each species could be readily determined by GC analysis of volatile FAME and DMA derivatives prepared from the corresponding column effluents.

Besides coelution of some species with different *sn*-1 and *sn*-2 aliphatic group composition, the resolution of 'reverse isomers' of diacylglycerophospholipids (i.e. phospholipid molecular species with identical aliphatic groups which differ in the location of each group at the *sn*-1 and *sn*-2 position (e.g. (16:0,18:1)PtdCho and (18:1,16:0)PtdCho)) also presents an important challenge to reversed-phase approaches for the separation of individual phospholipid molecular species. Using reversed-phase HPLC, we have previously shown that the 1-acyl and 2-acyl positional isomers of lysophosphatidylcholine may be readily separated using reversed-phase HPLC [15] demonstrating that the location of an aliphatic group at the *sn*-1 versus *sn*-2 position differentially affects the retention properties of phospholipid molecular species on a C_{18} column. Our results shown in Table 1 which demonstrate a 1.2-min difference in the retention time for the (18:1,18:2)PtdCho and (18:2,18:1)PtdCho 'reverse isomers' also suggest that it may be possible to resolve these species by

Table 2

Gradient elution reversed-phase HPLC separation of individual choline and ethanolamine glycerophospholipid molecular species from rabbit cardiac myocytes

Peak[a]	RRT[b]	Composition[c]	Effective total chain length[d]	Mol %[e]
	Choline glycerophospholipid molecular species			
a	0.540	(18:2,18:3)PtdCho	29.3	0.1±0.06
b	0.630	Unknown acid stable species		0.2±0.10
c	0.680	(18:2,20:4)PtdCho	30.2	0.4±0.15
d	0.736	(18:2,18:2)PtdCho*	30.5	2.1±0.20
e	0.897	(16:0,18:3)PlasCho	31.3	0.7±0.14
f	0.944	(16:0,20:4)PtdCho	31.4	5.1±0.31
g	1.000	(16:0,18:2)PtdCho*	31.7	16.1±0.64
h	1.051	(18:1,18:2)PtdCho	32.1	3.7±0.25
i	1.132	(16:0,20:4)PlasCho	32.3	9.1±0.35
j	1.210	(16:0,18:2)PlasCho	32.6	14.8±1.26
		(18:1,20:4)PlasCho	32.7	3.5±0.24
k	1.294	(16:0,18:1)PtdCho	32.8	19.3±1.54
l	1.355	(18:1,18:2)PlasCho	33.0	2.3±0.67
m	1.467	(18:0,20:4)PtdCho	33.4	4.1±0.30
n	1.542	(18:0,18:2)PtdCho	33.7	5.1±0.45
o	1.603	(16:0,18:1)PlasCho	33.7	6.4±0.51
p	1.740	(18:0,20:4)PlasCho	34.3	3.4±0.22
q	1.915	(18:0,18:2)PlasCho	34.6	1.9±0.28
r	2.423	(18:0,18:1)PlasCho	35.7	1.2±0.23
	Ethanolamine glycerophospholipid molecular species			
a	0.636	Unknown acid-stable species		0.2±0.11
b	0.687	(18:2,20:4)PtdEth	30.2	0.7±0.19
c	0.728	(18:2,18:2)PtdEth*	30.5	0.5±0.20
d	0.881	(16:0,18:3)PlasEth	31.3	2.3±0.46
e	0.940	(16:0,20:4)PtdEth	31.4	1.8±0.38
f	1.000	(16:0,18:2)PtdEth*	31.7	3.2±0.74
g	1.055	(18:1,18:2)PtdEth	32.1	1.8±0.14
h	1.138	(16:0,20:4)PlasEth	32.3	11.5±0.35
i	1.209	(16:0,18:2)PlasEth	32.6	0.5±0.21
		(18:1,20:4)PlasEth	32.7	13.3±0.83
j	1.279	(16:0,18:1)PtdEth	32.8	0.4±0.16
k	1.343	(18:1,18:2)PlasEth	33.0	2.8±0.52
l	1.426	(18:1,18:1)PtdEth*	33.2	0.6±0.3
m	1.457	(18:0,20:4)PtdEth	33.4	32.2±2.13
n	1.513	(18:0,18:2)PtdEth	33.7	7.7±0.48
o	1.586	(16:0,18:1)PlasEth	33.7	1.4±0.41
p	1.730	(18:0,20:4)PlasEth	34.3	10.1±0.74
q	1.890	(18:0,18:2)PlasEth	34.6	3.7±0.40
r	1.992	(18:0,18:1)PtdEth	34.8	3.1±0.21
s	2.407	(18:0,18:1)PlasEth	35.7	1.2±0.50

[a] Individual peaks are identified by the small case letters shown in Figs. 1 and 2.

[b] RRT=relative retention time. The RRT is defined as described in the text and in the legend to Table 1.

[c] The composition of individual phospholipid molecular species is given by the shorthand notation described in the legend to Table 1. Individual phospholipid molecular species were identified by GC analysis of the volatile FAME and DMA derivatives prepared by acid-catalyzed methanolysis as described in the text. The phospholipid mass of each species was determined by phosphate analysis of the corresponding column effluent. The unknown acid stable species could not be identified by GC analysis of volatile FAME and DMA derivatives. The acid stability of these species demonstrates that they are not plasmalogens (see text). Species identified by an asterisk (*) comprised 85–90% of the total mass in the corresponding column effluent but other coeluting species accounted for the remainder of the total phospholipid mass.

[d] Effective total chain length (ECL_T) was calculated as described in the text.

[e] Mol % represents the fractional contribution of each species to the total choline or ethanolamine glycerophospholipid mass. Rabbit cardiac myocytes contain approximately 40 nmol choline glycerophospholipid and 35 nmol ethanolamine glycerophospholipid per mg total cellular protein.

Table 3

Gradient elution reversed-phase HPLC separation of individual choline and ethanolamine glycerophospholipid molecular species from porcine endothelial cells

Peak[1]	RRT[2]	Composition[3]	Effective total chain length[4]	Mol %[5]
	Choline glycerophospholipid molecular species			
a	0.560	Unknown acid stable species		0.7±0.18
b	0.640	Unknown acid stable species		0.8±0.22
c	0.676	(18:2,20:4)PtdCho*	30.2	0.6±0.10
d	0.739	(18:2,18:2)PtdCho*	30.5	1.1±0.15
e	0.771	(16:0,18:3)PtdCho	30.5	1.4±0.26
f	0.792	Unknown acid stable species		2.3±0.22
g	0.865	Unknown acid stable species		1.3+0.23
h	0.884	Unknown acid stable species		3.7+0.18
i	0.940	(16:0,20:4)PtdCho	31.4	6.4±0.39
j	1.000	(16:0,18:2)PtdCho*	31.7	7.2±0.39
k	1.059	(18:1,18:2)PtdCho	32.1	4.1±0.20
l	1.140	(16:0,20:4)PlasCho	32.3	3.2±0.28
m	1.218	(16:0,18:2)PlasCho	32.6	2.0±0.56
		(18:1,20:4)PlasCho	32.7	3.8±0.44
n	1.304	(16:0,18:1)PtdCho	32.8	9.8±0.81
o	1.395	(16:0,18:2)AlkCho*	33.0	18.1±1.67
p	1.470	(18:0,20:4)PtdCho	33.4	12.4±0.98
q	1.550	(18:0,18:2)PtdCho	33.7	3.5±0.18
r	1.612	Unknown acid stable species		2.0±0.18
s	1.743	(18:0,20:4)PlasCho*	34.3	4.2±0.25
t	1.895	Unknown acid stable species		4.7±0.53
u	2.243	(18:0,18:1)PtdCho	34.8	6.6±0.38
	Ethanolamine glycerophospholipid molecular species			
a	0.643	Unknown acid-stable species		2.2±0.17
b	0.689	(18:2,20:4)PtdEth*	30.2	1.2±0.32
c	0.733	(18:2,18:2)PtdEth*	30.5	2.4±0.41
d	0.831	(18:1,18:3)PtdCho	30.9	2.9±0.20
e	0.890	(16:0,18:3)PlasEth*	31.3	3.3±0.38
f	0.946	(16:0,20:4)PtdEth	31.4	2.3±0.45
g	1.000	(16:0,18:2)PtdEth*	31.7	2.7±0.29
h	1.050	(18:1,18:2)PtdEth	32.1	3.2±0.32
i	1.143	(16:0,20:4)PlasEth	32.3	11.0±0.30
j	1.209	(16:0,18:2)PlasEth	32.6	1.7±0.41
		(18:1,20:4)PlasEth	32.7	9.5±0.83
k	1.283	(16:0,18:1)PtdEth	32.8	3.9±0.19
l	1.348	(18:1,18:2)PlasEth*	33.0	3.4±0.19
m	1.431	(18:1,18:1)PtdEth*	33.2	2.2±0.20
n	1.461	(18:0,20:4)PtdEth	33.4	13.1±0.27
o	1.584	Unknown acid stable species		4.3±0.11
p	1.626	Unknown acid stable species		3.6±0.14
q	1.780	(16:0,18:1)AlkEth*	34.4	3.5±0.19
r	1.860	(18:0,20:4)PlasEth	34.3	12.8±0.27
s	1.935	(18:0,18:2)PlasEth*	34.6	3.4±0.16
t	2.061	(18:0,18:1)PtdEth	34.8	1.9±0.17
u	2.351	Unknown acid stable species		4.0±0.46
v	2.427	(18:0,18:1)PlasEth	35.7	1.6±0.48

[a] Individual peaks are identified by the small case letters shown in Figs. 3 and 4.

[b] RRT=relative retention time. The RRT is defined as described in the text and in the legend to Table 1.

[c] The composition of individual phospholipid molecular species is given by the shorthand notation described in the legend to Table 1. Individual phospholipid molecular species were identified by GC analysis of the volatile FAME and DMA derivatives prepared by acid-catalyzed methanolysis as described in the text. The phospholipid mass of each species was determined by phosphate analysis of the corresponding column effluent. The unknown acid stable species could not be identified by GC analysis of volatile FAME and DMA derivatives. The acid stability of these species demonstrates that they are not plasmalogens (see text). Species identified by an asterisk (*) comprised 75–90% of the total mass in the corresponding column effluent but other coeluting species accounted for the remainder of the total phospholipid mass.

[d] Effective total chain length (ECL_T) was calculated as described in the text.

[e] Mol % represents the fractional contribution of each species to the total choline or ethanolamine glycerophospholipid mass. Porcine endothelial cells contain approximately 37 nmol choline glycerophospholipid and 34 nmol ethanolamine glycerophospholipid per mg total cellular protein.

reversed-phase HPLC. Further studies using multiple synthetic 'reverse isomers' are needed, however, to fully define the utility of reversed-phase HPLC for the separation of these isomeric diacylglycerophospholipids. For our studies shown in Figs. 1–4 and Tables 2 and 3, the assignment of the *sn*-1 and *sn*-2 aliphatic group composition of choline and ethanolamine diradylglycerophospholipids was based on the coelution of each species with synthetic phospholipid species of known *sn*-1 and *sn*-2 composition shown in Table 1. Our results are in agreement with previous studies of isolated canine cardiac myocytes [8] and myocardial sarcolemma [16] and sarcoplasmic reticulum [17] using regiospecific hydrolysis mediated by phospholipase A_2 and FAB MS to define the *sn*-1 and *sn*-2 aliphatic group composition. With these approaches, it is found that 18:1, 18:2, 18:3, 20:4 and 22:6 fatty acids comprise greater than 98% of the O-acyl ester-linked *sn*-2 aliphatic groups and that 93–95% of all *sn*-1 groups are saturated or monosaturated with 16 or 18 carbon atoms (18:2 groups accounted for most of the remaining 5–7%). For the diacylglycerophospholipids in the present study, it is important to point out that until further data is obtained to determine whether resolution of 'reverse isomers' can be achieved for all species, we cannot rule out the possibility that rabbit cardiac myocytes and porcine endothelial cells contain significant amounts of diacylglycerophospholipid molecular species with polyunsaturated groups at the *sn*-1 position and saturated or monounsaturated groups at the *sn*-2 position based on retention time data alone.

Comparison of the retention time data for individual choline and ethanolamine phospholipid species in Tables 2 and 3 demonstrates that the elution order and relative retention times are virtually identical for all species, thus, the composition of the polar headgroup does not significantly affect the retention characteristics for these two phospholipid classes. The relative retention times determined for the synthetic phospholipid molecular species shown in Table 1 agree to within ±5% of the relative retention times observed for corresponding choline and ethanolamine glycerophospholipids shown in Tables 2 and 3. This demonstrates the potential value of the relative retention time data to assist in the tentative identification of phospholipid species. It should also be noted that the calculated ECL_T based on the equation given above correctly predicts the order of elution for choline and ethanolamine glycerophospholipids.

4. Discussion

The present study demonstrates the advantages afforded by gradient elution separation of individual phospholipid molecular species as compared to isocratic approaches. With gradient elution, the elution sequence is not altered, however, improved resolution is obtained with the added advantage of a significant reduction in total elution time. The use of synthetic phospholipids to define the retention characteristics of individual molecular species provides unequivocal peak assignments and serves as a database of ART and RRT data which would be very helpful in providing a tentative identification of phospholipid species present in complex mixtures of phospholipids derived from biologic sources. Also, the ECL_T of an unidentified peak can be interpolated from the ECL_T values of known (identified) peaks eluting before and after the unknown peak. This interpolated ECL_T value provides a useful index which can also be used to facilitate the tentative identification of the unknown peak by comparison to the ECL_T values given in Table 1.

The definitive on-line identification and quantitation of individual phospholipid molecular species can, at present, only be achieved by HPLC coupled with MS utilizing thermospray (TS) or electrospray (ES) ionization techniques [18–20]. With these approaches, the separation of all individual molecular species is not critical since coeluting species can be distinguished on the basis of differences in molecular weight and mass fragmentation patterns of molecular ions or secondary ions generated in a tandem MS following collision-induced dissociation. Han and Gross, for example, were able to quantify over 50 different individual phospholipid molecular species using ES-MS with mass spectra acquired on a triple quadrapole tandem MS using a chloroform–methanol extract of erythrocyte phospholipids without prior chromatographic separation of any individual phospholipid molecular species [18]. Kim et al. [20] employed HPLC–ES-MS to analyze sphingo-

myelin and choline, serine, inositol and ethanolamine glycerophospholipids. These authors utilized gradient elution reversed-phase HPLC using a three component mobile phase containing methanol–hexane–0.5% ammonium hydroxide in water and were able to resolve several individual molecular species of PtdCho and PtdEth containing 16:0 or 18:0 at the *sn*-1 and 18:1, 20:4 or 22:6 at the *sn*-2 position. The (16:0,22:6)PlasEth and (18:0,22:6)PlasEth species were also resolved. Detailed information regarding the resolution of other phospholipid molecular species was not provided, however, with this ES-MS approach, resolution of individual species is only critical for isobaric species since mass-selective detection can be used. Although the present method was not specifically optimized for MS detection and quantitation of individual phospholipid molecular species, this approach should prove to be invaluable to those investigators who do not have access to sophisticated MS techniques for phospholipid quantitation. Our approach also provides a means to isolate individual species intact which is a distinct advantage over techniques utilizing MS which lead to destruction of the sample. Thus, the present method should be particularly useful for studies of the metabolism of individual phospholipid molecular species using radiolabeling techniques where isolation of the individual species is required prior to liquid scintillation counting.

There is an alternative approach to the isolation of phospholipid molecular species utilizing reversed-phase HPLC that is based on the separation of diradylglycerobenzoate derivatives of individual species formed following phospholipase C (PLC) catalyzed hydrolysis and reaction of the resulting diradylglycerol products with benzoyl chloride [5,21,22]. With this approach, resolution of individual molecular species is improved, however, there are several limitations: (1) conditions for hydrolysis and derivatization must be selected which result in quantitative conversion of all diacyl, alkenylacyl (plasmalogen) and alkylacyl species with no selectivity for hydrolysis of a specific subclass or selective loss during derivatization (2) additional time and effort must be expended for the hydrolysis, derivatization and isolation of the diradylglycerobenzoate product which also provides additional opportunity for oxidation and other undesirable covalent modification of sample molecules (3) the source of PLC must provide enzyme which is not contaminated by other phospholipases which could alter sample composition or by phospholipids which could accompany the enzyme during purification, (4) in radiolabelling experiments using [^{3}H]arachidonate, the radiolabeled diradylglycerobenzoate may eluate as a separate peak before the corresponding unlabeled species [5] and (5) with phospholipids radiolabeled at the *sn*-3 phosphobase group, the diradylglycerobenzoate approach cannot be used if measurements of both mass and radioactivity in isolated individual molecular species is desired. In addition, as described by Patton et al. [22], the 1,2-diradylglycerol product produced by PLC hydrolysis in biphasic ether–water mixtures at room temperature and neutral pH undergoes further intramolecular rearrangement to form 1,3-diradylglycerol species. After derivitization, the 1,2- and 1,3-diradylglycerobenzoate derivatives have different chromatographic retention characteristics, thus, biological samples containing complex mixtures of many different phospholipid molecular species may give rise to even more complex mixtures of 1,2- and 1,3-isomers that may be difficult or impossible to accurately quantify. For diacyl phospholipids, the 1,2-diacylglycerols undergo essentially quantitative rearrangement to 1,3-diacylglycerols. Accordingly, for samples comprised only of diacylglycerophospholipids, the PLC/diacylglycerobenzoate approach provides accurate quantitative assessment of the aliphatic composition of the diacylglycerophospholipid molecular species in the sample. However, for samples containing mixtures of diacylglycerophospholipids and ether-linked diradylglycerophospholipids, the ether linked phospholipids (alkylacyl and plasmalogen subclasses) do not undergo complete rearrangement and the result is a mixture of 1,2- and 1,3- diradylglycerol species [22] which obviously complicates any efforts to isolate and quantify these species in mixtures containing all three phospholipid subclasses. Isolation of the individual phospholipids as intact molecular species avoids most of these problems. Although we are unable to achieve complete resolution of all individual molecular species, it is possible to obtain accurate quantitative information by collection of peaks containing coeluting species followed by

quantitative GC analysis of volatile derivatives prepared from these species.

In addition to providing an approach to the quantitation of individual phospholipid molecular species, the present method is ideally suited for several additional applications including the isolation of individual phospholipid species for metabolic or functional studies, the preparation of homogeneous molecular species for studies of phospholipase substrate specificity, the isolation of single species as precursors for synthesis of specific labeled phospholipid derivatives and to permit turnover studies of individual phospholipid species in isolated cells after labelling with appropriate precursors. Accordingly, we anticipate that the present method for gradient elution reverse phase HPLC isolation of individual phospholipid molecular species should find widespread use.

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