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QUANTITATIVE ANALYSIS OF AMINOPHOSPHOLIPIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING SUCCINIMIDYL 2-NAPHTHOXYACETATE AS A FLUORESCENT LABEL

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SUMMARY

A simple and rapid high-performance liquid chromatographic procedure for the quantitative analysis of ethanolamine- and serine-containing phospholipids in tissue is described. The technique involves reaction of lipid extracts with succinimidyl 2-naphthoxyacetate to give fluorescent derivatives of aminophospholipids. Reaction products are separated by a silica gel column with gradient elution. The eluate is monitored by fluorescence detection at 228 nm (excitation) and 342 nm (emission). Ethanolamine and serine plasmalogens can be measured indirectly by converting their derivatives into lysophosphatidylethanolamine and lysophosphatidylserine derivatives with exposure to hydrogen chloride fumes. The method is highly sensitive and selective.

INTRODUCTION

Quantitative analysis of phospholipid composition in tissue extracts is frequently performed in biomedical research. A simple, rapid and accurate method for this analysis is highly desirable. Thin-layer chromatography (TLC) separates phospholipid classes but is tedious and requires additional assays for quantitation of fractions. Recently, several investigators [1—5] have developed high-performance liquid chromatographic (HPLC) methods for phospholipid analysis. These methods are adequate for the separation of many major and minor components. However, the use of ultraviolet (UV) detection does not allow direct quantitation of fractions, because the 200-nm range of phospholipid absorbance reflects the number of double bonds rather than the number of molecules [1]. HPLC with flame ionization detection [6] has been used to quantitate phospholipid fractions, but it is relatively insensitive.

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Ethanolamine- and serine-containing phospholipids can be easily converted into N-acyl derivatives prior to HPLC analysis. If the detection is aimed at the chromatophore introduced by derivatization, the peak area on the chromatogram reflects the amount of phospholipid eluted. This appears to be a convenient way of measuring the concentrations of different aminophospholipid classes. Thus Jungalwala et al. [7] have analyzed aminophospholipids as their biphenylcarbonyl derivatives by HPLC with UV detection at 280 nm. In order to improve the sensitivity we have previously converted aminophospholipids into Dns derivatives and monitored their HPLC separation with fluorescence detection [8]. In this report we describe the analysis by HPLC of aminophospholipids as their naphthyl derivatives. Compared with the analysis using Dns chloride [8], the method described here has the same sensitivity and specificity but offers the advantage of speed. The reaction time for making naphthyl derivatives is shorter and the HPLC using the new gradient elution program is twice as fast.

EXPERIMENTAL

Materials

Egg yolk phosphatidylethanolamine (PE), egg yolk lysophosphatidylethanolamine (LysoPE), bovine brain phosphatidylserine (PS), bovine brain lysophosphatidylserine (LysoPS) and N-succinimidyl 2-napththoxyacetate were purchased from Sigma (St. Louis, MO, U.S.A.). The purity of phospholipids was checked by TLC. All solvents were of reagent grade. [Dipalmitoyl-1-^{14}C]-phosphatidylethanolamine was purchased from New England Nuclear (Boston, MA, U.S.A.).

Rat brain lipid extract

Sprague—Dawley male rats weighing 150 g were used. Immediately after decapitation heads were placed in liquid nitrogen. A 1-g amount of tissue was removed from the frozen brain and homogenized in 30 ml of chloroform—methanol (2:1). After filtration the lipid extract was separated into two phases according to the procedure of Folch et al. [9]. An aliquot of the lower phase was used for derivatization.

Derivatization of aminophospholipids

An aliquot of lipid solution (phospholipid standards or the total lipid extract from rat brain), containing less than 3 μg of lipid phosphorus, was transferred to a 12 × 32 mm vial (Catalogue No. 223682; Wheaton Scientific, Millville, NJ, U.S.A.). The solvent was evaporated at 50°C under nitrogen. To the dried lipids 5 μl of triethylamine were added, followed by 45 μl of succinimidyl naphthoxyacetate (1 mg/ml in chloroform, freshly prepared). A 3-mole excess of the reagent over aminophospholipids was sufficient for derivative formation as indicated by the yield and recovery studies (see Results). Vials were tightly capped by aluminum seals with a crimper, and vortexed vigorously for 10 sec. They were shaken in the dark at room temperature for 2 h. After incubation, samples were either analyzed immediately or vials were stored at −20°C.

Phospholipid derivatives are stable for several days at this temperature. Because triethylamine might interfere with the PE peak on the chromatogram, just before HPLC analysis reaction products in the vial were evaporated to dryness under nitrogen at 50°C and redissolved in a small amount of chloroform. An aliquot was taken and injected onto the chromatograph.

HPLC analysis

We used a Waters Assoc. (Milford, MA, U.S.A.) Model 6000 solvent delivery system combined with a Model 660 solvent programmer and a Model U6K injector. The chromatographic column was a 30 cm × 4 mm I.D. prepacked stainless-steel Mikro-Pak SI-10 column (Varian, Palo Alto, CA, U.S.A.), which contained silica gel, particle size 10 μm. The column was initially equilibrated with solvent A [dichloromethane—methanol—15 *M* ammonium hydroxide (90:11:1.5)]. The separation of phospholipid derivatives was carried out by programmed gradient elution as follows: flow-rate 1.5 ml/min, 5 min with linear gradient from 100% solvent A to 100% solvent B [dichloromethane—methanol—15 *M* ammonium hydroxide (70:20:5)], and 10 min with solvent B. The gradient program started at the time the sample was injected onto the chromatograph. Before the next analysis, the column was regenerated to its original polarity by equilibrating it with solvent A for 5 min or more. The column temperature was that of room temperature, 21°C.

Fluorescence detection

The column effluent was monitored by fluorescence detection with excitation and emission wavelengths of 228 and 342 nm, respectively. The slit width was 10 nm. We used a Perkin-Elmer Model MPF-44B fluorescence spectrophotometer equipped with an HPLC flow cell, part No. 063-0575. Chromatograms were recorded on a Model 057 x-y recorder. Peak areas were calculated by a Model 9874 digitizer interfaced with a Model 9830A calculator (Hewlett-Packard, Palo Alto, CA, U.S.A.). Uncorrected excitation and emission spectra of PE derivative were obtained by a stop-flow technique, i.e., spectra were scanned while the derivative was trapped in the flow cell by stopping the flow of eluent.

Hydrolysis of ethanolamine and serine plasmalogens

The total lipid extract was derivatized. The reaction mixture was dried under nitrogen. The open vial was then inverted and held over an open bottle of concentrated hydrochloric acid for 10 min. After flushing the vial with nitrogen, chloroform was added in an amount identical with that of the original sample. An aliquot was injected into the chromatograph for analysis.

RESULTS

Fluorescence spectra

Uncorrected spectra of PE derivative showed three peaks of excitation at 228 nm, 272 nm and 320 nm (Fig. 1). The emission maximum was 342 nm. Similar spectra were obtained from PS derivative. Either 228 nm or 272 nm can be used as the excitation wavelength for the fluorescence detection of amino-

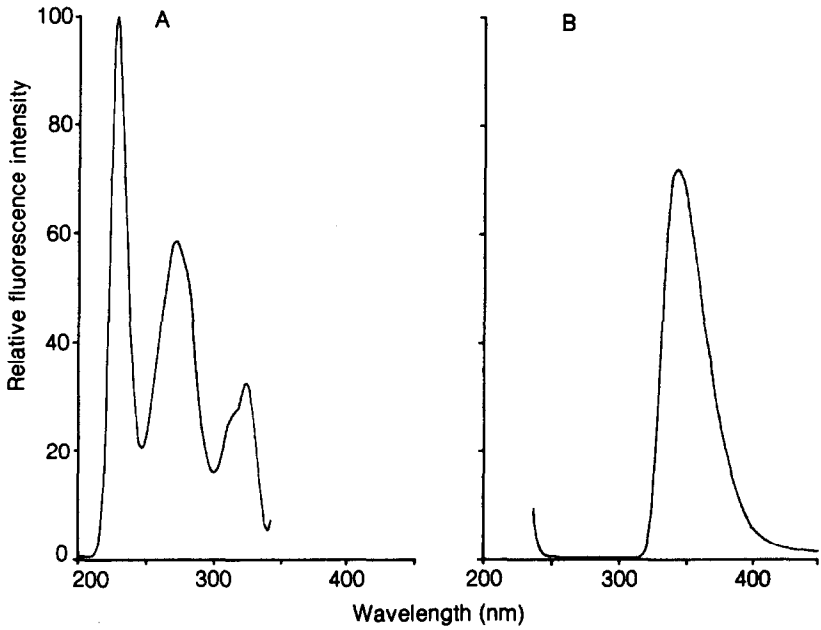

Fig. 1. Uncorrected excitation (A) and emission (B) spectra of phosphatidylethanolamine derivative in dichloromethane—methanol—15 *M* ammonium hydroxide (90:11:1.5).

phospholipid derivatives. The excitaiton at 228 nm gave slightly greater response and was used in this study.

Gradient elution program

Several solvent mixtures containing dichloromethane—methanol—15 *M* ammonium hydroxide in various proportions were tested for their ability to elute derivatives of aminophospholipids. With isocratic elution it was not possible to separate all four derivatives rapidly, because the polarity of these compounds is quite different. The gradient elution program shown in Fig. 2 could efficiently separate all four derivatives in a single run within 15 min. Retention times of PE, LysoPE, PS and LysoPS derivatives were 5, 7.5, 11, and 13 min, respectively. Fluorescent peaks due to impurities of reagents and reaction by-products did not interfere with the analysis. For example, 2-naphtoxyacetic acid is located at peak c (Fig. 2). The identity of peaks on the chromatogram was established by injecting into the chromatograph separately the reaction product prepared from the individual phospholipid standard.

Derivatization conditions

Aminophospholipids reacted readily with succinimidyl 2-napthoxyacetate at room temperature. As shown in Fig. 3, the reaction time to reach a maximal and constant fluorescence response was within 1 h. In this present study, 2 h at room temperature was used as the standard condition. To evaluate the yield of derivatization and the recovery of HPLC analysis we determined the phosphorus content, with a micro colorimetric method [10], in the original phospholipid standard solution before derivatization and in the fluorescent

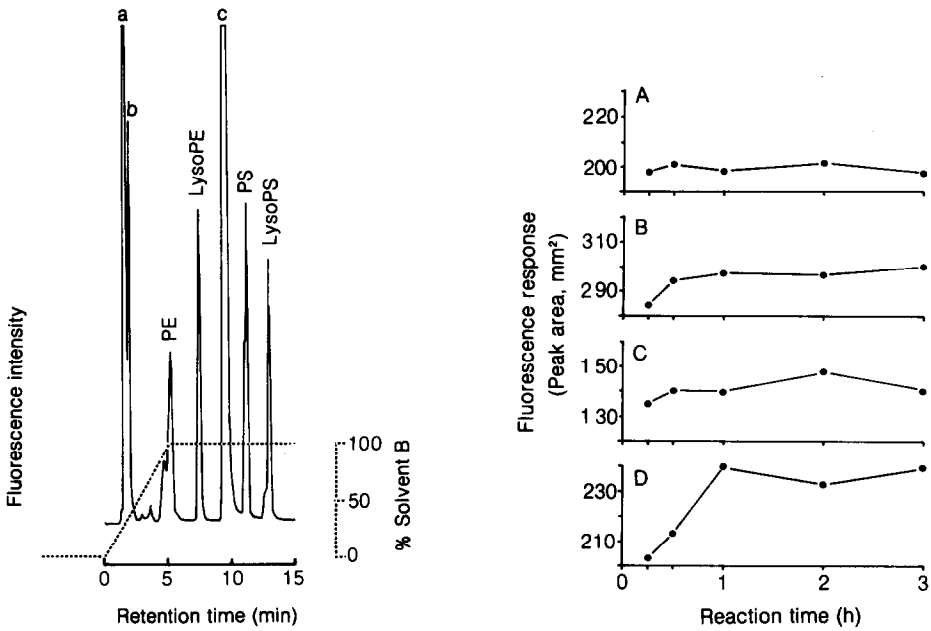

Fig. 2. Chromatogram of derivatives of phospholipid standards: phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LysoPE), phosphatidylserine (PS) and lysophosphatidylserine (LysoPS). Peaks a, b and c are due to reaction by-products and impurities of reagents. The aliquot contained 10 ng lipid phosphorus of each lipid. The elution was with a gradient of dichloromethane—methanol—15 *M* ammonium hydroxide from solvent A (90:11:1.5) to solvent B (70:20:5). The flow-rate was 1.5 ml/min.

Fig. 3. Influence of reaction time on derivatization of (A) phosphatidylethanolamine, (B) lysophosphatidylethanolamine, (C) phosphatidylserine and (D) lysophosphatidylserine. N-Succinimidyl 2-naphthoxyacetate was added to vials of phospholipid soluton which contained PE, LysoPE, PS and LysoPS. At 15 min, 30 min, 1 h, 2 h and 3 h after derivatization vials were open and analyzed by HPLC.

peak collected from HPLC. Recoveries of PE, LysoPE, PS and LysoPS were 95, 92.5, 93 and 81%, respectively (average of two determinations). The quantitative recovery of PE was also confirmed by counting the radioactivity in the PE derivative peak when a known amount of [dipalmitoyl-1-^{14}C]phosphatidylethanolamine (18,000 cpm) was derivatized and an aliquot was injected onto the chromatograph.

Standard curves

The fluorescence intensity was linear with respect to concentration (Fig. 4). Standard curves of PE and LysoPE, in terms of peak area per nmole of phospholipid, overlapped each other. Ethanolamine-containing phospholipids (PE and LysoPE) showed slightly greater fluorescence response per nmole of phospholipid than serine-containing phospholipids (PS and LysoPS). Although the amount of phospholipids injected in the experiment shown in Fig. 4 was in the nmole range, the present method is exquisitely sensitive and is suitable for measuring phospholipid quantities in the pmole range. The detection limit was 2 pmoles of phospholipid (60 pg of lipid phosphorus) which showed a signal-to-noise ratio of 2:1 on the chromatogram.

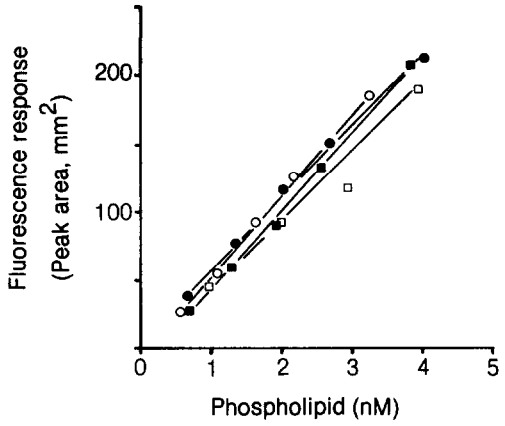

Fig. 4. Standard curves for four phospholipid derivatives: phosphatidylethanolamine (•), lysophosphatidylethanolamine (○), phosphatidylserine (▪) and lysophosphatidylserine (□). Known amounts of phospholipid standards were derivatized and injected for HPLC analysis under the same conditions as in Fig. 2. The fluorescence response in terms of peak area due to each phospholipid was measured.

Quantitative analysis of rat brain aminophospholipids

The usefulness of the present method for the quantitative analysis of aminophospholipids in tissue extracts is illustrated in Fig. 5 and Table I. The total

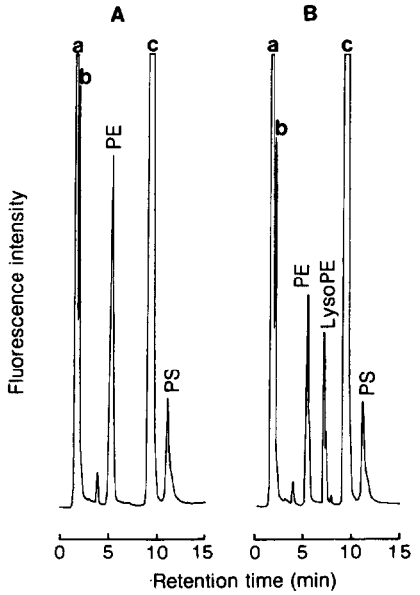

Fig. 5. HPLC analysis of derivatives of the total lipid extract of rat brain before (A) and after (B) exposure to hydrogen chloride fumes. The total lipid extract, containing 1 μg of lipid phosphorus, was derivatized as described in the Experimental section. An aliquot of the reaction mixture was injected into the chromatograph. Another aliquot was dried, exposed to hydrogen chloride fumes, redissolved in chloroform and then injected. The analysis was by gradient elution as described in Fig. 2. Peaks: PE = Phosphatidylethanolamine; lysoPE = lysophosphatidylethanolamine; PS = phosphatidylserine; a, b and c = reaction by-products and impurities of reagents.

TABLE I

PHOSPHOLIPID COMPOSITION OF RAT BRAIN

	Present analysis*	HPLC by Chen et al. [8]	HPLC by Chen and Kou [5]	HPLC by Jungalwala et al. [7]
Before exposure to HCl fumes				
PE	40.7 ± 0.5	43.6 ± 1.2	40.0 ± 2.8	41.6 ± 2.6
LysoPE	None detected		None	
PS	12.7 ± 0.2	17.1 ± 0.4		11.7 ± 1.6
LysoPS	None detected			
After exposure to HCl fumes				
PE	20.4 ± 1.8		19.2 ± 2.0	
LysoPE (derived from plasmalogens)	18.6 ± 0.9	18.9 ± 0.6	19.9 ± 0.9	22.9 ± 1.3

*The values in the present analysis, percentages of the total phospholipids, are mean ± S.D. obtained from three rats.

lipid extract from rat brain containing 1 μg of lipid phosphorus was derivatized. An aliquot was injected into the chromatograph. For the analysis of ethanolamine and serine plasmalogens another aliquot of the reaction mixture was exposed to hydrogen chloride fumes before HPLC analysis in a manner similar to that described by Jungalwala et al. [7]. This is based on previous observations that hydrogen chloride fumes quantitatively hydrolyze alk-1-enyl group from phosphoglycerides and neutral glycerides [11, 12]. The chromatogram of the original lipid extract (Fig. 5A) reveals no detectable amounts of lysophosphatidylethanolamine and lysophosphatidylserine in rat brain. The exposure to hydrogen chloride fumes converted the ethanolamine plasmalogen derivative into a lysophosphatidylethanolamine derivative, since Fig. 5B shows that the peak corresponding to PE derivative decreased, while a peak corresponding to LysoPE derivative appeared. From peak areas in the chromatogram we calculated the quantities of phosphatidylethanolamine, ethanolamine plasmalogen (converted to lysophosphatidylethanolamine derivative) and phosphatidylserine in rat brain by reference to standard curves obtained from known amounts of phospholipids. In Table I data are compared with those obtained by using other HPLC methods. The results were in good agreement. Day-to-day precision of the analysis was evaluated by measuring aliquots of same lipid extracts for four times over a period of 18 days. Three lipid extracts were analyzed. Coefficients of variation (standard deviation/mean) averaged 8%, 18%, and 11% for PE, PS, and ethanolamine plasmalogen, respectively.

DISCUSSION

N-Succinimidyl 2-naphthoxyacetate has been previously used for the detection of amino acids on paper chromatograms [13]. The present method is the first use of this reagent for labelling ethanolamine- and serine-containing

phospholipids. Using rat brains we showed that the method was applicable to the analysis of phospholipids containing these amino groups in tissue samples. It appears to offer several advantages over TLC and other HPLC methods. The most important of these is probably the saving in time and labor, since the derivatization is easy to perform and the HPLC analysis is rapid. If tissue extracts do not contain detectable amount of LysoPE or LysoPS, one of these commercially available lipids can be added to Folch lipid extracts and used as an internal standard. This will improve the speed and the accuracy of quantitation. The sensitivity of measurement is another striking feature. Trace amounts of phospholipids in the pmole range can be determined. Also noteworthy about the method is the specificity introduced by derivatization, chromatographic separation and fluorescence detection. It is free from interfering compounds that might cause error in the analysis.

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