Journal of Chromatography, 208 (1981) 339–346
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,589

MEASUREMENT OF ETHANOLAMINE- AND SERINE-CONTAINING PHOSPHOLIPIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION OF THEIR Dns DERIVATIVES

S. SHI-HUA CHEN*, ANNE Y. KOU and HSIN-HSIN Y. CHEN
Pathology Department, Stanford University and Palo Alto Veterans Medical Center, Stanford, CA 94305 (U.S.A.)
(Received October 23rd, 1980)

SUMMARY

We describe a liquid chromatographic procedure for the analysis of amino group-containing phosphoglycerides in tissue. The total lipid extract is derivatized with Dns-chloride at 50°C for 3 h. Dns derivatives of phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylserine and lysophosphatidylserine are separated by a silica gel column with gradient elution. The eluate is monitored by fluorescence detection at 342 nm (excitation) and 500 nm (emission). Ethanolamine and serine plasmalogens can be determined indirectly by converting their derivatives into Dns-lysophosphatidylethanolamine and Dns-lysophosphatidylserine with exposure to HCl fumes. The optimal sample size for derivative formation and analysis by this method is between 1 and 10 nmol of phospholipids (30 and 300 ng of lipid phosphorus), although the lower limit of detection is about 20 pmol. By analyzing the total lipid extract of rat brain we showed that the method was applicable to the quantitative analysis of amino group-containing phosphoglycerides in tissue samples.

INTRODUCTION

Currently the popular procedure for quantitative analysis of tissue phospholipids requires extraction of total lipids, separation into individual phospholipids by column and/or thin-layer chromatography (TLC) and colorimetric determination of phosphorus content. The procedure is complex, time consuming and difficult to be precise. Attempting to develop a faster and more efficient procedure several investigators[1–3] have previously explored the possibility of measuring phospholipids by high-performance liquid chromatography (HPLC). When phospholipids are analyzed directly by HPLC, one of the difficulties is in the detection. Phospholipids do not absorb UV light around 254 nm. They can be detected at 203 nm where double bonds and other functional groups absorb energy. However, the choice of solvents becomes limited, because solvents must be transparent in the 203-nm region. Two solvent systems have been previously used as eluents in the direct analysis of phospholipids

0021-9673/81/0000–0000/$02.50

by HPLC with UV detection at 203 nm[1,2]. Unfortunately, neither of them affords a clear separation of all of the major phospholipids. An alternative to direct analysis is to make derivatives of phospholipids before the analysis. Thus Jungalwala *et al.*[3] have analyzed ethanolamine- and serine-containing phospholipids as their biphenylcarbonyl derivatives by HPLC with UV detection at 280 nm. 1-Dimethylaminonaphthalene-5-sulfonyl chloride (Dns-Cl) reacts readily with primary and secondary amines to form fluorescent derivatives. In conjunction with TLC this fluorogenic labelling technique has been applied successfully to the analysis of a wide range of organic compounds for several years[4]. Dns-Cl reacts also with phospholipids that contain primary amino groups[5]. In this report we show that the combined use of the Dns derivatization technique, HPLC and fluorescence detection offers a sensitive and specific method for the quantitative analysis of ethanolamine- and serine-containing phospholipids in tissue.

EXPERIMENTAL

Materials

Egg yolk phosphatidylethanolamine, egg yolk lysophosphatidylethanolamine, bovine brain phosphatidylserine, bovine brain lysophosphatidylserine and Dns-Cl were purchased from Sigma (St. Louis, MO, U.S.A.). The purity of phospholipids was checked by TLC. All solvents were of reagent grade.

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.*[6]. An aliquot of the lower phase was used for Dns derivatization.

Dns derivatization

An aliquot of lipid solution (phospholipid standards or the total lipid extract from rat brain), containing less than 1 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 8 μl of triethylamine was added, followed by 25 μl of Dns-Cl solution (1 mg/ml in chloroform, freshly prepared). Vials were tightly capped by aluminum seals with a crimper, and vortexed vigorously for 10 sec. They were incubated in the dark in a 50°C water bath for 3 h. After incubation, samples were either analyzed immediately or vials were stored at −20°C. Phospholipid derivatives are stable for at least 24 h at this temperature.

Gradient elution HPLC analysis

Just before HPLC analysis, the vial containing the reaction mixture was opened and a 3.5-μl aliquot was injected directly into the chromatograph. 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* NH_4OH; 91:9:1). The separation of Dns derivatives was carried out by programmed gradient elution as follows (Fig. 1): flow-rate 1 ml/min, 5 min with solvent A, 10 min with linear gradient from 100% solvent A to 100% solvent B (dichloromethane–methanol–15 *M* NH_4OH; 70:20:5), and 15 min with solvent B Before the next analysis, the column was regenerated to its original polarity by equilibrating it with solvent A for 15 min. The column temperature was that of room temperature, 21°C.

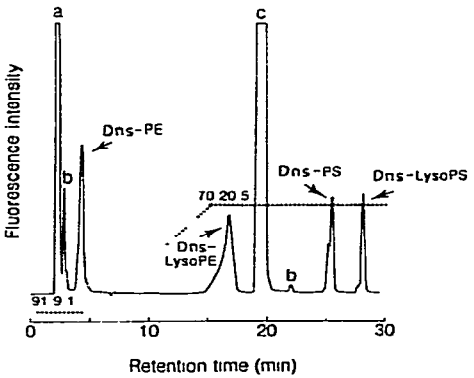

Fig. 1. Chromatogram of Dns derivatives of phospholipid standards: phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LysoPE), phosphatidylserine (PS) and lysophosphatidylserine (LysoPS) Dns-Cl and Dns-NH_2 are located in peak "a", while peak "c" is Dns-OH Two peaks labelled as "b ' are unidentified components The amount injected was 3 5 μl of the Dns derivatization mixture containing 0 2 μg of each phospholipid. The elution was with a 10-min linear gradient of dichloromethane–methanol–15 *M* NH_4OH from 91.9:1 to 70 20.5.

Fluorescence detection

The column effluent was monitored by fluorescence detection with excitation and emission wavelengths of 342 and 500 nm, respectively. The slit width was 10 nm. The signal gain setting was 0.3. 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 Dns-phosphatidylethanolamine was obtained by a stop-flow technique, *i.e.*, spectra were scanned while the Dns derivative was trapped in the flow cell by stopping the flow of eluent.

Hydrolysis of ethanolamine and serine plasmalogens

The procedure described by Jungalwala *et al.*[3] for converting Dns derivatives of these compounds into Dns-lysophosphatidylethanolamine and Dns-lysophosphatidylserine was followed. 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 conc. HCl 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.

Preparation of Dns-NH_2 and Dns-OH

An aliquot of the reaction mixture of phosphatidylethanolamine Dns derivatization was applied to a silica gel H TLC plate and developed for 1 h with chloroform–methanol–15 *M* NH_4OH (12:6:1). Fluorescing bands of Dns-NH_2 and Dns-OH were identified with a UV lamp, since their color and R_F values are distinctive[4]. Silica gel bands are scrapped and eluted with chloroform.

RESULTS

Fluorescence spectra

Uncorrected excitation and emission spectra of Dns-phosphatidylethanolamine in dichloromethane–methanol–15 *M* NH_4OH (91:9:1) showed that the excitation wavelength maximum was 342 nm and the emission maximum was 500 nm (Fig. 2). Similar results were obtained from Dns-phosphatidylserine.

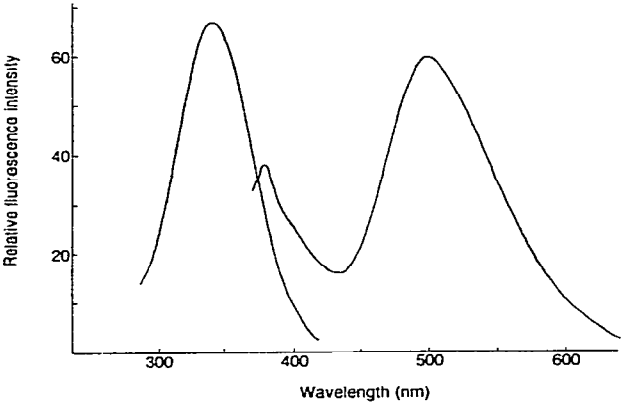

Fig 2. Uncorrected excitation (left) and emission (right) spectra of Dns-phosphatidylethanolamine in dichloromethane–methanol–15 *M* NH_4OH (91 9:1). Spectra were obtained with a stop-flow technique (see Experimental).

Development of a gradient elution program

Several solvent systems, including dichloromethane–methanol–acetic acid–water and dichloromethane–methanol–15 *M* NH_4OH in various proportions, were tested for their ability to separate the four Dns derivatives of phospholipids. With isocratic elution it was not possible to analyze all four derivatives with a single injection, because the polarity of these compounds is quite different. The elution of Dns-phosphatidylethanolamine required a less polar solvent system. On the other hand, a more polar solvent system is needed to elute the other three Dns phospholipids and Dns-OH in a short period of time and as sharp peaks. Therefore, we developed a gradient elution program which could efficiently separate all four phospholipids and Dns-OH in a single run. Fig. 1 shows that retention times of Dns-phosphatidylethanolamine, Dns-lysophosphatidylethanolamine, Dns-OH, Dns-phosphatidylserine and Dns-lysophosphatidylserine were 4, 15, 19, 25 and 27 min,

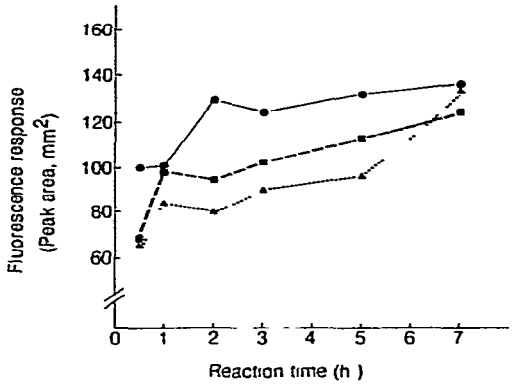

Fig. 3. Influence of temperature and reaction time on phosphatidylserine Dns derivatization Phosphatidylserine (4.7 μg in each vial), Dns-Cl (25 μl) and triethylamine (8 μl) were incubated at 22°C (▲), 37°C (■) or 50°C (●) At various times the vial was chilled in ice and one tenth of the sample was injected for HPLC analysis The elution was isocratic with dichloromethane–methanol–15 *M* NH_4OH (80 15 3) The fluorescence response due to Dns-phosphatidylserine was measured as peak area

respectively. The asymmetry of peaks probably is due to the heterogeneity in the fatty acid composition of phospholipids. The identity of peaks on the chromatogram was established by injecting into the chromatograph separately the reaction mixture prepared from the individual phospholipid standard. Dns-NH_2 and Dns-OH are usual by-products of Dns derivatization. In order to be certain about their locations on the chromatogram, purified Dns-NH_2 and Dns-OH were eluted from TLC plate and injected into the chromatograph (see Experimental).

Derivatization conditions

The time course of the Dns derivatization of phosphatidylserine was investigated at 22, 37 and 50°C. At various times after incubation the reaction mixture was injected into the chromatograph. The fluorescence response due to the product, Dns-phosphatidylserine, was measured. As shown in Fig. 3, the reaction time to reach a maximal response was temperature dependent. Maximal response was obtained at 50°C by 2 h, and at 22 and 37°C for 7 h. In this present study Dns derivatization at 50°C for 3 h was used as the standard condition. To evaluate the yield of Dns derivatization and recovery of analysis we determined the phosphorus content, with a micro colorimetric method[7], in the original phospholipid standard before Dns derivatization and in the Dns-phospholipid peak collected from HPLC. The recovery of phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylserine and lysophosphatidylserine were 85.5 ± 1.0, 91.7 ± 2.0, 85.4 ± 2.7, and 63.4 ± 5.1, respectively (mean ± S.D., $n = 4$). A better yield of Dns derivatization presumably can be obtained by using a larger quantity of Dns-Cl in the reaction mixture. However, increasing the amount of Dns-Cl broadens peaks of Dns-Cl, Dns-NH_2 and Dns-OH and may interfere with the analysis.

Standard curve

The concentration of phospholipid standards was determined with Bartlett

method[8]. Varying quantities of standards were then derivatized and analyzed by HPLC. The relationship between peak area and the quantity of standard is shown in Fig. 4. The linearity of response with the amount of phospholipid injected was evident. Since one tenth of the reaction mixture was injected into the chromatograph, between 1 and 10 nmol of phospholipid (30 and 300 ng of lipid P) is the optimal sample size for derivative formation and analysis by this procedure, although the lower limit of detection is about 20 pmol.

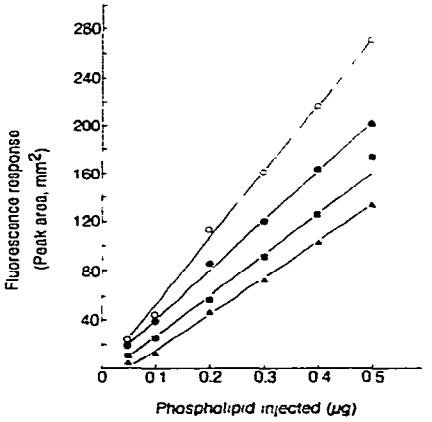

Fig. 4 Standard curves for four phospholipid derivatives: phosphatidylethanolamine (●), lysophosphatidylethanolamine (○), phosphatidylserine (■) and lysophosphatidylserine (▲). Known amounts of phospholipid standards were derivatized and injected for HPLC analysis under the same conditions as in Fig 1. The fluorescence response in terms of peak area due to each phospholipid was measured.

Quantitative analysis of rat brain phospholipids

The total lipid extract from rat brain, containing 7.5 μg of phospholipids (10 nmol, or 300 ng of lipid P), 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 HCl fumes before HPLC analysis in a manner similar to that described by Jungalwala *et al.*[3]. This is based on previous observations that HCl fumes quantitatively hydrolyze alk-1-enyl group from phosphoglycerides and neutral glycerides[9,10]. The chromatogram of the original lipid extract (Fig. 5a) reveals no detectable amounts of lysophosphatidylethanolamine and lysophosphatidylserine in rat brain. The exposure to HCl fumes converted the ethanolamine plasmalogen derivative into a lysophosphatidylethanolamine derivative, since Fig. 5b shows that the peak corresponding to Dns-ethanolamine phosphoglycerides decreased, while a peak corresponding to Dns-lysophosphatidylethanolamine 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 a standard curve obtained from known amounts of phospholipid standards. In Table I our data are compared with those obtained by other investigators[3,11,12]. The results of phosphatidylethanolamine and ethanolamine plasmalogen determinations were in good agreement. Our phosphatidylserine value was slightly higher.

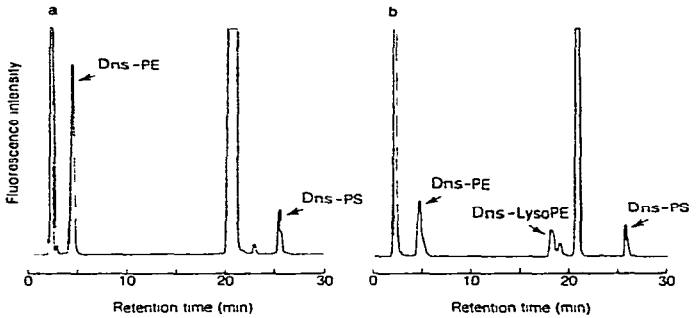

Fig. 5. HPLC analysis of Dns derivatives of the total lipid extract of rat brain before (a) and after (b) exposure to HCl fumes. The total lipid extract, containing 300 ng of lipid P, was derivatized as described in the Experimental section. An aliquot of the reaction mixture was injected directly into the chromatograph Another aliquot was dried, exposed to HCl fumes, redissolved in chloroform and then injected The analysis was by gradient elution as described in Fig. 1 PE = Phosphatidylethanolamine; lysoPE = lysophosphatidylethanolamine; PS = phosphatidylserine.

TABLE I
PHOSPHOLIPID COMPOSITION OF RAT BRAIN

	*Present analysis**	*HPLC by Jungalwala et al.*[3]	*TLC by Cuzner et al*[11]	*TLC by Norton and Poduslo*[12]
Phosphatidylethanolamine	43 6 ± 1 2	41.6 ± 2 6	41.1	32 2
Ethanolamine plasmalogens	18 9 ± 0.6	22 9 ± 1 3	22 3	17 4
Phosphatidylserine	17.1 ± 0 4	11 7 ± 1 6	11 6	12 3

* The values in the present analysis, percentages of the total phospholipids, are mean ± S D obtained from 3 rats.

DISCUSSION

This paper describes a method by which amino group-containing phosphoglycerides in tissues can be measured directly from the total lipid extract. The method is specific and exquisitely sensitive. The results of analysis of rat brain by this method are in general agreement with those reported by other investigators. Therefore, the method is ideal for the micro analysis of amino group-containing phospholipids in minute amounts of tissue. Jungalwala *et al.*[3] have previously analyzed ethanolamine- and serine-containing phosphoglycerides by HPLC with UV detection at 280 nm of their biphenylcarbonyl derivatives. In comparison with their method, our method offers the following advantages. First, the sample preparation is simple, since the reaction mixture is injected directly into the chromatograph without prior purification of reaction product. Purification is not necessary because of the specificity of fluorescence detection. Secondly, fluorescence detection is more sensitive than UV detection. The amount of sample in the optimal range for analysis with fluorescence detection is about 10-fold smaller than that with UV detection. Thirdly, the gradient elution program described here efficiently separate Dns derivatives of phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylserine and lysophosphatidylserine in a single run. These phospholipids appear on the same chromatogram and

can be analyzed simultaneously. With the method described by Jungalwala *et al.*[3] two separate injections are required for the analysis of phosphatidylethanolamine, lysophosphatidylethanolamine and phosphatidylserine. Lysophosphatidylserine was not identified in their study.

ACKNOWLEDGEMENT

This investigation was supported by a grant from the Veterans Administration.

REFERENCES

1 F. B Jungalwala, J. E. Evans and R. H. McCluer, *Biochem J.*, 155 (1976) 55.
2 W. S M Geurts van Kessel, W. M A. Hax, R. A. Demel and J. de Gier, *Biochim. Biophys Acta*, 486 (1977) 524.
3 F. B. Jungalwala, R. J. Turel, J. E. Evans and R. H. McCluer, *Biochem J*, 145 (1975) 517.
4 N. Seiler, *Methods Biochem. Anal.*, 18 (1970) 259.
5 A. S. Waggoner and L. Stryer, *Proc. Nat. Acad. Sci U S.*, 67 (1970) 579.
6 J. Folch, M Lees and G H. S. Stanley, *J. Biol. Chem*, 226 (1957) 497
7 A Chalvardjian and E. Rudnicki, *Anal. Biochem.*, 36 (1970) 225.
8 G. R. Bartlett, *J. Biol. Chem*, 234 (1959) 466
9 H. H. O. Schmid and H. K. Mangold, *Biochim. Biophys Acta*, 125 (1966) 182
10 L. A. Horrocks, *J. Lipid Res*, 9 (1968) 469.
11 M. L. Cuzner, A. N. Davison and N. A Gregson, *J. Neurochem*, 12 (1965) 469
12 W. T. Norton and S. E. Poduslo, *J. Neurochem.*, 21 (1973) 759.