Journal of Chromatography, 307 (1984) 261—269
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in the Netherlands

CHROMBIO. 2035

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PHOSPHOLIPIDS USING DEUTERATED SOLVENTS FOR INFRARED DETECTION

S. SHI-HUA CHEN*,* and ANNE Y. KOU

Department of Pathology, Stanford University and Palo Alto Veterans Administration Medical Center, Stanford, CA 94305 (U.S.A.)

(First received September 19th, 1983; revised manuscript received December 8th, 1983)

SUMMARY

Infrared detection of chromatographic effluents offers the advantage of direct on-line quantitation of lipid fractions. However, infrared detection imposes limitations on the solvent systems that can be used for chromatography. Methanol and water, which are essential ingredients in the mobile phase for the successful chromatography of phospholipids, do not have spectral transmittance windows in the infrared region. Substituting deuterated methanol and deuterium oxide for methanol and water allowed infrared detection because they had lower infrared absorbance than their hydrogenated counterparts. We report a method that is suitable for the quantitative analysis of phosphatidylethanolamine, phosphatidylcholine and sphingomyelin in the tissue extracts. The lipid separation was accomplished on a micropraticulate silica gel column. Phosphatidylethanolamine and phosphatidylcholine were eluted isocratically with chloroform—acetonitrile—methanol—deuterium oxide (136:25:34:5.9) and detected at a wavelength of 5.75 μm. For the analysis of sphingomyelin, chloroform—acetonitrile—deuterated methanol—deuterium oxide (130:24:37.6:7.0) was used as the mobile phase, and the detection was at a wavelength of 6.15 μm.

INTRODUCTION

The traditional procedure for quantitative analysis of phospholipid composition in tissues involves extraction of total lipids, separation into lipid

*Address for correspondence: Laboratory Service (113), Veterans Administration Medical Center, 3801 Miranda Avenue, Palo Alto, CA 94304, U.S.A.

0378-4347/84/$03.00

classes by either thin-layer or column chromatographic methods, and quantitation by measuring the phosphorus content. The procedure is tedious and imprecise. Attempting to develop a simpler procedure, several investigators [1—8] have explored the possibility of using high-performance liquid chromatography (HPLC). HPLC provides fine separation of lipids. However, because of the problem of detection a satisfactory HPLC technique for direct quantitation of lipid classes is not yet available. The most popular method of detection is based on the change in ultraviolet (UV) absorbance. Being sensitive and non-destructive, UV absorbance detection is ideal for monitoring the separation of lipids by HPLC. But, it 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]. Usually the effluents from specific peaks are collected into the tubes and quantified by colorimetric methods. Detectors based on the principle of refractive index are relatively insensitive and incompatible with gradient elution techniques. Moving wire flame ionization detector allows direct quantitation of lipid fractions [7, 8]. However, it is also insensitive and the bulk of sample is destroyed by the flame. Since this type of detector lacks widespread popularity, they are no longer manufactured commercially.

Most lipids have specific structures that exhibit strong absorption bands in the infrared (IR) region of the spectrum. IR absorbance detection offers considerable potential for direct on-line quantitation of lipid fractions. It has been applied to the HPLC of neutral lipids [9, 10]. The major disadvantage is that it imposes limitations on the solvent systems that can be used in chromatography. Only a few solvents are transparent at the wavelengths where lipids absorb. Despite the limited choice of solvents, we have developed an HPLC technique using IR detection for the quantitative analysis of several major phospholipids in tissue extracts. The novelty of the technique is the use of deuterated methanol and deuterium oxide as substitutes for methanol and water in the mobile phase. The substitution clears the obstacle that methanol and water, essential ingredients for the chromatography of phospholipids, are not transparent in the spectral region of interest.

EXPERIMENTAL

Materials

Reference compounds were purchased from Sigma (St. Louis, MO, U.S.A.). Phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), phosphatidylcholine (PC), lysophosphatidylcholine (LPC) and sphingomyelin (SPH) were derived from egg yolk. Phosphatidylserine (PS) was from bovine brain, whereas phosphatidylinositol (PI) was from soybean. Deuterium oxide (99.8 atom % ^{2}H) and deuterated methanol (99.5 atom % ^{2}H) were obtained also from Sigma. Chloroform, acetonitrile and methanol were of HPLC grade from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). [Dipalmitoyl-1-^{14}C]-phosphatidylethanolamine and [dipalmitoyl-1-^{14}C] phosphatidylcholine (100 mCi/m*M*) were purchased from New England Nuclear (Boston, MA, U.S.A.).

Tissue lipid extracts

Sprague--Dawley male rats weighing 150 g were used. They had access to the diet up to the time of sacrifice. Immediately after decapitation, heads and livers were placed in liquid nitrogen. Plasma was from a healthy human donor. A 1-g amount of rat tissue from cerebrum, right lobe of liver or 1 ml of human plasma was 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. [11]. The upper phase was discarded and the lower phase was washed once with reconstituted pure solvent upper phase [11]. Lipid phosphorus in tissue extracts was measured by the Bartlett procedure [12].

Instruments

We used a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatographic system consisting of a Model 6000 solvent delivery system and a Model U6K injector. The column was a 30 cm × 3.9 mm I.D. prepacked μPorasil column (Waters Assoc.) which contained silica gel, particle size 10 μm. There was a guard column packed with Corasil (Waters Assoc.). The liquid chromatograph infrared detector was a DuPont product (Wilmington, DE, U.S.A.). The optical path length of the calcium fluoride cell was 1 mm and the internal cell volume was 463 μl (Analabs, Cat. No. 006-7026, North Haven, CT, U.S.A.). Chromatograms were recorded on a strip chart recorder. Peak areas were calculated by a Model 9874 digitizer interfaced with a Model 9830A calculator (Hewlett-Packard, Palo Alto, CA, U.S.A.).

Chromatographic conditions and IR detection

Phospholipid reference compounds and Folch [11] tissue lipid extracts were dried under nitrogen in a warm water bath. They were redissolved in a small amount of mobile phase, and an aliquot was injected onto the chromatograph. Sample sizes are indicated in figure legends. The elution was isocratic. For the analysis of PE and PC the solvent was chloroform—acetonitrile—methanol—deuterium oxide (136:25:34:5.9) and the detection was at a wavelength of 5.75 μm. For the analysis of sphingomyelin the solvent was chloroform—acetonitrile—deuterated methanol—deuterium oxide (130:24:37.6:7.0) and the detection wavelength was set at 6.15 μm. The mobile phase was delivered to the column at a flow-rate of 1 ml/min. The pressure was approximately 103 bars. The column temperature was that of the room temperature, 21°C. The IR detector was set at 2-mm slit width and 4 response meter. The range was either 0.1 A or 0.025 A as indicated in the figures.

RESULTS

IR detection

The separation of phospholipid classes, except for sphingomyelin, was monitored by IR detection at a wavelength of 5.75 μm, which corresponds to the absorbance of carbonyl group. The detection of sphingomyelin was based on the absorbance of amide group at 6.15 μm. Some of the best solvents for lipophilic samples, e.g., chloroform and acetonitrile, have spectral transmittance windows in these wavelength regions. Water and methanol are not IR

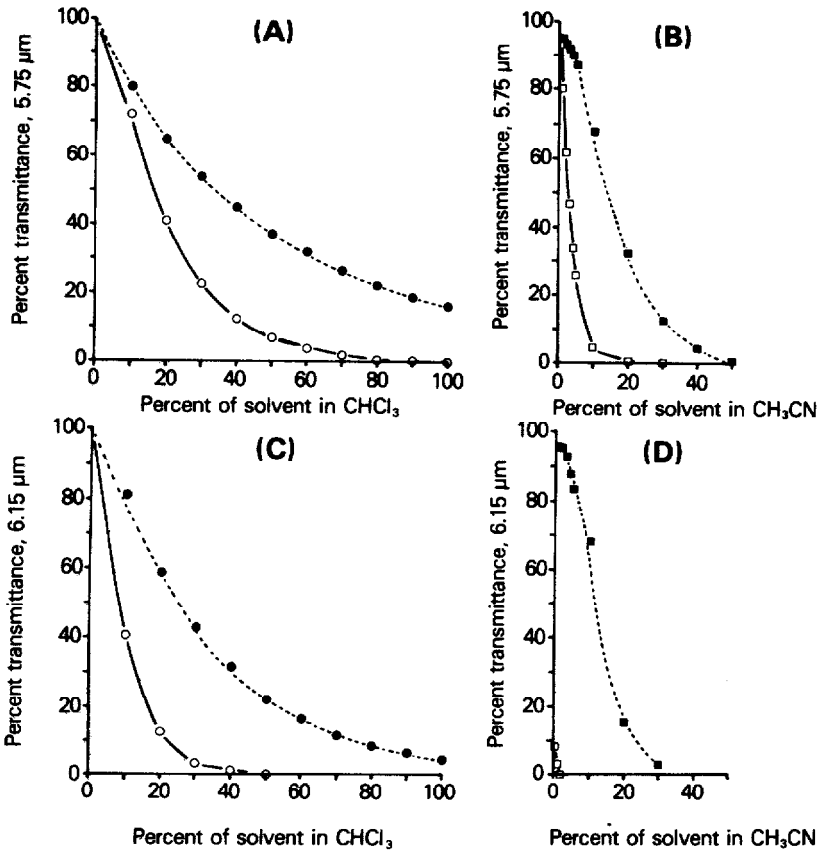

Fig. 1. (A) and (C) IR transmittance of deuterated methanol (•- - -•) and methanol (○—○) in chloroform; (B) and (D) IR transmittance of deuterium oxide (▪- - -▪) and water (□—□) in acetonitrile. In (A) and (B) the IR transmittance was measured at a wavelength of 5.75 μm, whereas in (C) and (D) the wavelength was set at 6.15 μm.

transparent, but they are essential ingredients in the mobile phase for the successful chromatography of phospholipids. Water absorbs the light at 5.75 μm and 6.15 μm so strongly that its presence even in a small percentage was incompatible with IR detection. In order to overcome this difficulty, deuterium oxide was used to replace water in the mobile phase. Deuterium oxide and deuterated methanol do not have spectral transmittance windows in the 5.75-μm and 6.15-μm regions. But, their IR absorbances were much less than those of their hydrogenated counterparts (Fig. 1). For IR detection at 5.75 μm the substitution of deuterated methanol for methanol was usually unnecessary, since the solvent (chloroform—acetonitrile—methanol—deuterium oxide, 136:25:34:5.9) allowed sufficient transmission of light for the detector to operate. The use of deuterated methanol could reduce the noise level and improve the detection sensitivity, but it is relatively expensive. For the detection of sphingomyelin at 6.15 μm deuterated methanol must be used to replace methanol in the mobile phase.

HPLC of major phospholipid classes

Because phospholipids vary greatly in polarity and charges, our attempt to develop a solvent system that could separate all the major classes isocratically in a single run was unsuccessful. Gradient elution method was not used, because it introduced a steep baseline which complicated the calculation of peak areas. We found a solvent system suitable for the analysis of PE and PC. Fig. 2 shows the chromatograms of reference compounds and various tissue extracts using this solvent system (chloroform—acetonitrile—methanol—deuterium oxide, 136:25:34:5.9). PE and PC were rapidly eluted and well separated from other lipids. Neutral lipids (NL) and diphosphatidylglycerol (DPG) were eluted with the solvent front. Sphingomyelin was not expected to be detectable at 5.75 μm, because the detection was based on the absorbance of carbonyl group. No other sharp peaks were detected on the chromatograms, even though PS, PI and LPC were present. Based on the phosphorus analysis of HPLC fractions, PS, PI, SPH and LPC were eluted during the following time intervals: PS from 12 to 17 min; PI from 15 to 20 min; SPH from 15 to 18 min; and LPC from 17 to 19 min. Evidently the mobile phase was not polar enough to resolve these lipids into sharp peaks. Poor detector sensitivity was another reason that phospholipids in small quantities might not be detectable. The appearance of a biphasic peak coincided with the completion of phospho-

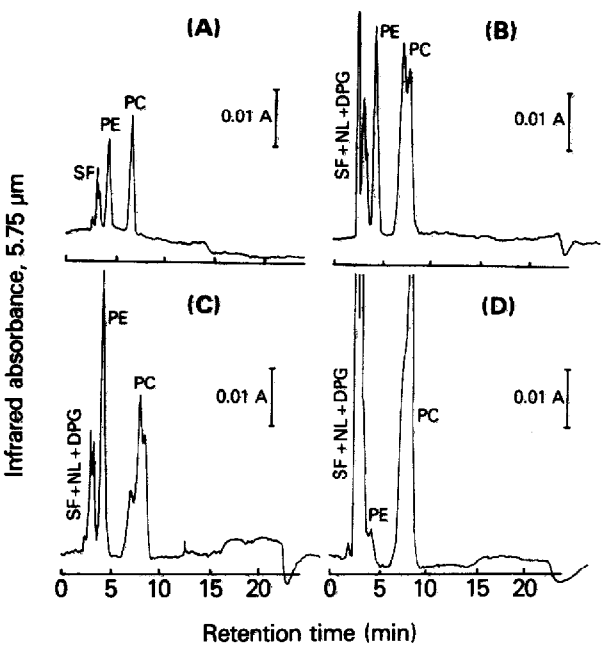

Fig. 2. HPLC analysis of reference compounds and tissue extracts. (A) Phospholipid standards; (B) rat liver; (C) rat brain; and (D) human plasma. The isocratic elution was with chloroform—acetonitrile—methanol—deuterium oxide (136:25:34:5.9), and the detection wavelength was set at 5.75 μm. The flow-rate was 1 ml/min. Other conditions were described under Experimental. The sample in chromatogram A contained 200 μg each of PE, PC, SPH, PS, PI and LPC. Aliquots of the total lipid extracts in B, C and D contained approximately 1.5 mg of total phospholipids. Peaks: SF = solvent front; NL = neutral lipids; DPG = diphosphatidylglycerol; PE = phosphatidylethanolamine; and PC = phosphatidylcholine.

lipid elution. The mechanism for this phenomena is unclear. For the analysis of sphingomyelin we employed chloroform—acetonitrile—deuterated methanol—deuterium oxide (130:24:37.6:7.0) as the mobile phase (Fig. 3). Sphingomyelin co-eluted with PS, but the presence of PS did not interfere with IR detection at 6.15 μm, because the absorbance was specifically due to sphingomyelin. It is noteworthy that in Fig. 3 the chromatograms show a partial separation of two fractions. The pattern was similar to the result obtained with silica gel thin-layer chromatography (TLC). It was previously reported that in the TLC of sphingomyelin the more rapidly moving component contained primarily C_{22} and C_{24} fatty acids, whereas the slower component contained mainly palmitic acid [13].

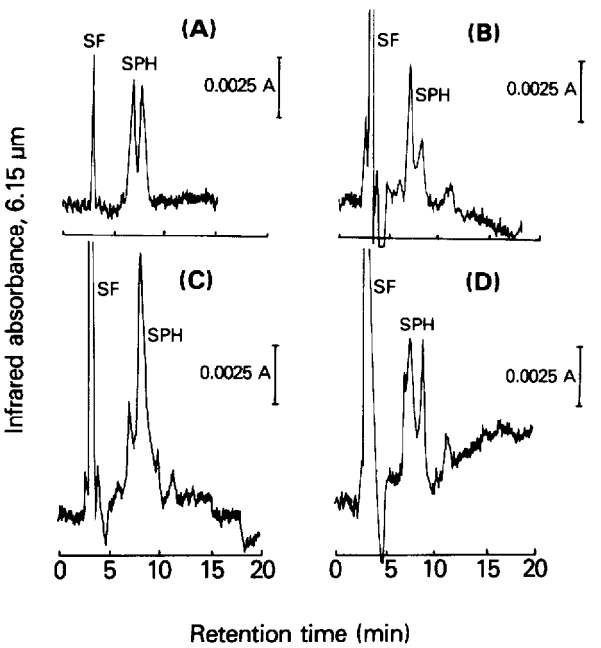

Fig. 3. HPLC analysis of reference compound and tissue extracts. (A) Egg yolk sphingomyelin, 200 μg; (B) rat liver; (C) rat brain; and (D) human plasma. Aliquots of total lipid extracts in B, C and D contained approximately 2 mg of total phospholipids. The isocratic elution was with chloroform—acetonitrile—deuterated methanol—deuterium oxide (130:24:37.6:7.0), and the detection wavelength was set at 6.15 μm. The flow-rate was 1.0 ml/min and the range of detector was set at 0.025 A. Peaks: SF = solvent front; SPH = sphingomyelin.

In the analysis of tissue lipid extracts, identification of peaks in Figs. 2 and 3 was accomplished by comparing retention times of lipid standards, by TLC analysis of individually collected fractions and by analyzing the IR spectra of the collected fractions.

The recoveries of phospholipids applied to the column were determined by the analysis of phosphorus content in the collected fractions. [^{14}C]PE and [^{14}C]PC were used both to evaluate the recoveries and to confirm the identity of peaks. The recoveries of DPG, PE, PC, PS, PI, SPH and LPC were respectively 94, 94, 93, 78, 84, 88 and 76% (average of at least three determinations for each phospholipid).

Standard curves

The standard curves relating peak area to concentration are shown in Fig. 4. The linearity of response between 30 and 250 n*M* phospholipid was evident. On a molar basis PE had a lower IR response than PC. This was related to the fact that the PE peak was rich in ethanolamine plasmalogen. Since the detection was based on the absorbance of carbonyl group, ethanolamine plasmalogen had a molar response factor of only one, whereas phosphatidylethanolamine had two. The PC peak also contained some choline plasmalogen but the proportion was very small.

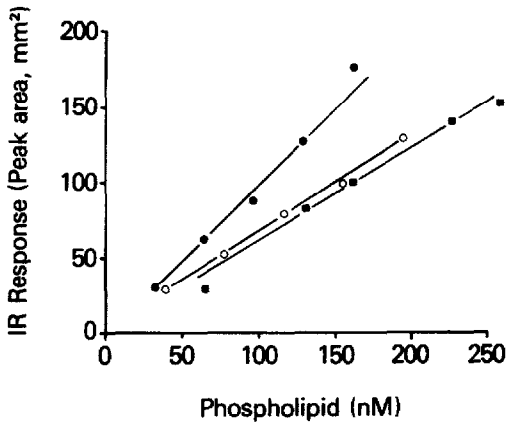

Fig. 4. Standard curves for phosphatidylethanolamine (○—○), phosphatidylcholine (●—●) and sphingomyelin (■—■). Known amounts of phospholipid standards were injected for HPLC analysis and the IR response in terms of peak area due to each phospholipid was measured.

Analysis of rat liver phospholipids

The method described here is suitable for the quantitative analysis of phospholipids in tissue extracts. An example is the analysis of PE, PC and SPH in rat liver (Table I). PE and PC determined by the IR peak area method agreed well with the results obtained by determining the amount of phosphorus in the eluted peaks. They were also in agreement with those results reported in the literature [14]. Within-run variability of the peak area method was small. Coefficients of variation (C.V.; S.D./mean) for PE, PC, and SPH were 0.5, 3.5 and 4.5% respectively. Day-to-day variations were 6.8% for PE, 6.1% for PC and 13.1% for SPH.

DISCUSSION

The major difficulty in using IR detection arises from restrictions on selecting solvents. In order to increase the sensitivity and minimize the noise level, the mobile phase should have a low absorbance at the wavelength employed. Phospholipid separation by a silica gel column requires the use of polar solvents. Unfortunately most polar solvents, such as methanol and water, do not have reasonable spectral transmittance windows in the IR region. In this present study we demonstrated that the problem of solvent absorbance could be greatly lessened by using deuterated compounds. The approach of

TABLE I

WITHIN-RUN AND DAY-TO-DAY PRECISION FOR THE DETERMINATION OF PER CENT COMPOSITION OF PHOSPHOLIPIDS IN RAT LIVER

For the analysis of PE and PC, aliquots of total lipid extracts from the rat liver, each containing 1 μM total phospholipids, were injected into the chromatograph as described in Fig. 2B. For the analysis of SPH, each aliquot of total lipid extracts contained 2 μM total phospholipids, and the chromatography was the same as that described in Fig. 3B. The values quoted, percentages of the total phospholipids, were determined by either the peak area method or measuring the phosphorus content in the collected peak effluents.

Phospholipids	Within-run (n = 3)				Day-to-day (n = 3)			
	Peak area method		Phosphorus assay		Peak area method		Phosphorus assay	
	Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)
PE	23.7	0.5	25.4	3.4	25.1	6.8	25.0	4.4
PC	53.7	3.5	50.4	2.7	50.6	6.1	50.1	2.0
SPH	3.3	4.5	—	—	3.6	13.1	—	—

using deuterated compounds expands the list of solvents that can be used for chromatography.

Another possible approach towards solving the problem of solvent restriction is to improve the design and the sensitivity of IR spectrophotometers. With conventional detectors it is preferable to use a mobile phase having over 30% transmittance at specified wavelength settings. Some specially designed detectors reportedly are so sensitive in detecting the solutes that solvents having only 5% transmittance can be used [15].

We have previously developed several HPLC methods for the analysis of phospholipids using either fluorescence or UV detection [3—6]. Each of these methods has its applications and limitations. Compared with UV detection, IR detection has the following advantages. First, since the detection is based on the absorbance due to either carbonyl or amide group, it imparts a degree of specificity to the detector response. Secondly, peak areas on the chromatogram reflect the amount of phospholipids eluted from the column. With reference to standard curves, direct quantitation of lipid classes can be rapidly accomplished. It should be emphasized that the PE standard employed should have the same proportion of ethanolamine plasmalogen as the samples tested. This is because in the chromatography ethanolamine plasmalogen co-elutes with phosphatidylethanolamine. Since the detection is based on the absorbance of carbonyl group, the response of the PE peak varies with the concentration of ethanolamine plasmalogen. If tissue extracts do not contain detectable amounts of lysoPE, this commercially available lipid can be added to the Folch [11] lipid extracts and used as an internal standard. This will improve the accuracy and the speed of analysis. Using the solvent mixture described in Fig. 2, the retention time of lysoPE is 13 min.

Disadvantages of IR detection include poor sensitivity and solvent restriction. The smallest amount of PE, PC or SPH we could measure was about 30 nM. The optimal sample size for the analysis was approximately 1 μM of

total phospholipids in the tissue extracts (31 μg of lipid phosphorus). This is about 100 times as large as the sample size for UV analysis. Sensitivity of analysis is a factor that minor phospholipids could not be detected. Solvent restriction hinders the development of a mobile phase that can satisfactorily separate PS, PI, SPH, and LPC from each other. Although PS co-elutes with sphingomyelin in chromatography, the method is still valid for the quantitation of sphingomyelin because of the specificity of detection at 6.15 μm.

In conclusion, this present study shows the potential that IR can be used for direct on-line quantitation of chromatographic effluents. The method described here is suitable for either the quantitation or the isolation of PE and PC from tissue extracts. The chromatography is rapid and free from other contaminants. However, the usefulness of this present method for the analysis of minor phospholipids appears limited. More work in improving the detection sensitivity and in the selection of columns and solvent systems is required in order to develop a satisfactory method for the analysis of minor phospholipids.

ACKNOWLEDGEMENT

This investigation was supported by the Veterans Administration.

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