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# High-Performance Liquid Chromatography of Phosphatidylcholine and Sphingomyelin with Detection in the Region of 200nm

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A sensitive method for the separation of phosphatidylcholine and sphingomyelin by high-performance liquid chromatographic analysis is described. The elution of the phospholipids from a microparticulate (10$\mu$m) silica-gel chromatographic column was monitored with an ultraviolet spectromonitor at 203nm. Acetonitrile/methanol/water (65:21:14, by vol.) was used as the solvent. It was shown by using synthetic phosphatidylcholines of known fatty acid composition and of varying degree of unsaturation that the absorption at 203nm was primarily due to the isolated double bonds and the response measured varied with the degree of unsaturation. Approx. 1nmol of phosphatidylcholine, containing at least one double bond per molecule, can be detected. The amounts of phosphatidylcholine and sphingomyelin could be determined by high-performance liquid chromatography and ultraviolet absorption if the apparent extinction coefficient of the material analysed was established. Alternatively, peaks were collected and the phospholipids were determined by the analysis of phosphorus. The analysis of phosphatidylcholine and sphingomyelin present in the lipid extracts from animal tissues, blood and amniotic fluids were made without interference from other phospholipids or ultraviolet-absorbing material. The method described here is complementary to the high-performance liquid chromatographic method described previously for the analysis of ethanolamine-containing phosphoglycerides and serine-containing phosphoglycerides [Jungalwala, Turel, Evans and McCluer (1975) *Biochem. J.* **145**, 517–526].

Until recently the successful application of modern h.p.l.c.* with ultraviolet detection has been primarily limited to materials that absorb ultraviolet light around 254 or 280nm, because commercially available minimum-volume flow-detectors operated only at these two fixed wavelengths. We have previously described a sensitive method for the separation and quantitative measurement of phosphatidylethanolamine, ethanolamine plasmalogens, phosphatidylserine and lysophosphatidylethanolamine by h.p.l.c. (Jungalwala *et al.*, 1975). In this method, the phospholipids containing primary amino groups are easily and quantitatively converted into their biphenylamides. These phospholipid derivatives, with molar extinction coefficients of about 20000 at 280nm, are separated and estimated by h.p.l.c. Phosphatidylcholine and sphingomyelin do not have reactive functional groups that could be easily subjected to derivative formation. Recently, variable-wavelength ultraviolet flow-monitors have become available. This has prompted us to explore the possibility of the direct detection of phospholipids and other complex lipids in the near ultraviolet region where many different functional groups including isolated double bonds are known to absorb energy. This report describes the h.p.l.c. of phosphatidylcholine and sphingomyelin with detection at 203nm and its application to the analysis of tissue samples and body fluids. Part of this work has been reported previously in an abstract (McCluer *et al.*, 1975).

* Abbreviation: h.p.l.c., high-performance liquid chromatography.

## Experimental

### *Instrumentation*

The h.p.l.c. analysis was performed with a Waters Associates (Milford, MA, U.S.A.) model 6000 solvent delivery system combined with a solvent programmer model 660. The chromatographic column was of 50cm×2.1mm (internal diameter) stainless-steel pre-packed with silica gel (Micropak, SI-10; Varian, Palo Alto, CA, U.S.A.). The average particle diameter of the silica gel was 10$\mu$m. Detection was with a Schoeffel Instruments Corp. (Westwood, NJ, U.S.A.) model SF 770 variable-wavelength spectromonitor coupled to a strip-chart recorder. The spectromonitor has two 8$\mu$l flow cells. The peak areas were measured by cutting the paper and weighing, unless stated otherwise.

*Materials*

Acetonitrile and methanol, the solvents used for chromatography, were of ultraviolet spectral grade (Burdick Jackson Laboratories, Muskegon, MI, U.S.A.), and have cut-offs below 200nm. Synthetic distearoyl-, dioleoyl-, dilinoleoyl-, dilinolenoyl- and diarachidonyl-L-α-glycerylphosphorylcholines were from either Supelco Inc., Bellefonte, PA, U.S.A., or Serdary Research Laboratories, London, Ont., Canada. Phosphatidylcholine (bovine) and sphingomyelin (bovine) were from Supelco Inc. [U-$^{14}$C]-Phosphatidylcholine was obtained from New England Nuclear, Boston, MA, U.S.A.

*Tissue lipid extracts*

The lipids from liver, brain and human erythrocyte samples were extracted by the method of Jungalwala *et al.* (1975). The lipids from the amniotic fluid samples were extracted as follows. The amniotic fluid (about 2ml) was centrifuged at 700*g* for 10min at 0°C to remove suspended cells. The supernatant was mixed with 2.7ml of methanol followed by 5.4ml of chloroform. The mixture was centrifuged at room temperature (24°C) at 1000*g* for 5min. The upper phase was discarded and the lower phase was washed once with 5ml of methanol/0.9% (w/v) NaCl/chloroform (48:47:1, by vol.). The lower phase was dried under $N_2$, and the lipids were dissolved in 100μl of ethanol. Phosphorus analyses were made according to the method of Bartlett (1959) as modified by Dittmer & Wells (1969). T.l.c. of phospholipids was performed by the method of Horrocks (1968).

## Results

*Chromatographic conditions*

The h.p.l.c. analysis of bovine phosphatidylcholine and sphingomyelin is shown in Fig. 1. A sample consisting of 10nmol of bovine phosphatidylcholine and 16.6nmol of sphingomyelin dissolved in ethanol was injected into the column. The acetonitrile/methanol/water (65:21:14, by vol.) solvent was delivered to the column at a flow rate of 1ml/min at a pressure of approx. 0.218N·m$^{-2}$ (1500lb.in$^{-2}$), at room temperature (24°C). The recorder response was set at 0.1 absorbance unit per full-scale deflexion at 203nm. The reference cell contained air. Baseline separations were achieved, and the analysis was complete within 15min after the injection of the sample. The same column and chromatographic conditions were used repeatedly for several months without any loss of reproducibility. When the chromatographic resolution deteriorated the column was washed with methanol and dichloromethane successively to regenerate its full activity. In some cases (Fig. 5) a less polar solvent was used to achieve better

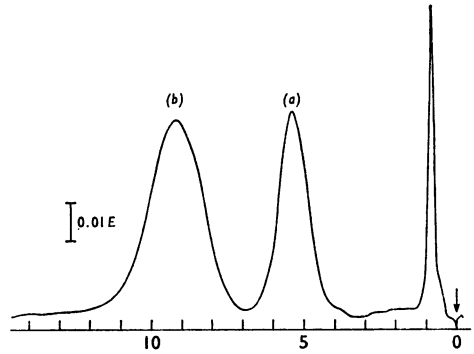


Fig. 1. *High-performance liquid chromatography of bovine brain phosphatidylcholine (a) and sphingomyelin (b) standards*

Chromatographic conditions were: elution solvent, acetonitrile/methanol/water (65:21:14, by vol.); flow rate, 1ml/min; column adsorbant, MicroPak SI-10. Detection was at 203nm. The arrow represents the point of injection. This is a representative example of six analyses of phosphatidylcholine and sphingomyelin standards.

resolution of phosphatidylcholine from other lipids eluted near the solvent front. The polarity of the solvent was varied, without affecting the resolution of phosphatidylcholine and sphingomyelin, by changing the proportion of acetonitrile and keeping the methanol/water ratio at 3:2. Whenever a new solvent of the same or different composition was used the adsorbent was re-equilibrated by pumping the new solvent through the column. The column was considered re-equilibrated and ready for sample analysis after the retention times of the standards had stabilized. We have also used a preparative column (50cm×4.6mm) for the separation of mg quantities of phospholipids under similar conditions. The theoretical plates of the column under the conditions described was found to be around 500 and the plate height was 1mm.

*Recovery*

[U-$^{14}$C]Phosphatidylcholine (approx. 3300d.p.m.) was mixed with non-radioactive phosphatidylcholine and injected under the conditions described. The radioactivity recovered in the phosphatidylcholine peak was in the range of 95–100%. The identification and recoveries of phosphatidylcholine and of sphingomyelin were also confirmed by t.l.c. and phosphorus analysis of the material eluted from the column. The recovery of both these compounds was 95–100% by phosphorus analysis. Only one spot corresponding to the peak of phosphatidylcholine and sphingomyelin was seen by t.l.c. after spraying the plates with aq. 40% $H_2SO_4$ and charring.

*Quantification and absorption spectra*

The percentage relative standard deviation of the mean for six repeated injections of the same sample, as in Fig. 1 (10nmol of phosphatidylcholine or 16.6nmol of sphingomyelin per injection), was 0.5–0.8%. For all subsequent analyses, each sample was injected 2–3 times and the peak area averaged. It was observed that the variations in the peak area between injections of the same samples was within the range quoted.

Fig. 2 (*a* and *b*) show that a linear ultraviolet response, measured in terms of peak area, could be obtained for quantities of 1–60$\mu$g of phosphatidylcholine and 1–40$\mu$g of sphingomyelin. Samples in 5–100$\mu$l of ethanol were injected. The retention times were independent of the sample quantities.

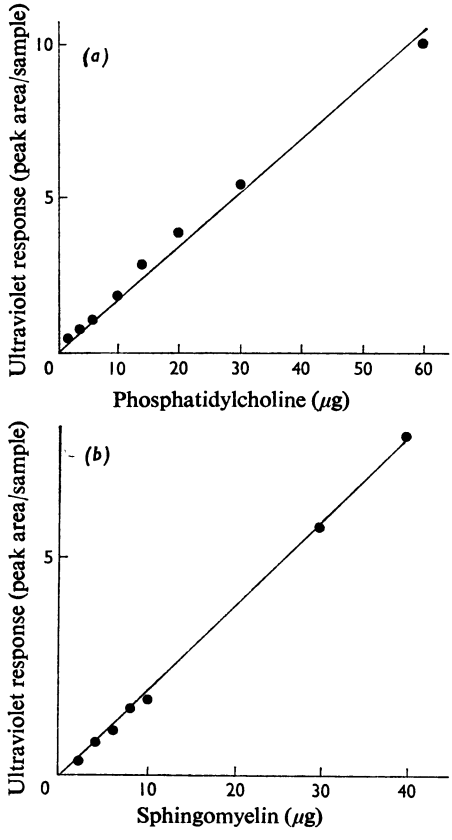


Fig. 2. *Quantitative analysis of bovine brain phosphatidylcholine (a) and sphingomyelin (b) standards by high-performance liquid chromatography*

The amounts of phospholipids indicated were injected and the ultraviolet response was measured in terms of peak area. Other conditions were the same as in Fig. 1. The points represent the average of two independent observations.

The absorption spectra of the bovine brain phosphatidylcholine and sphingomyelin were determined by repeated injection of the same amount of the lipid and by measurement of the response in terms of absorbance units at the peak maximum, at different wavelength settings from 198 to 212nm (Fig. 3). The maximum response, with the instrument used, was at 203nm. This maximum is instrument-dependent, since the amount of stray light is greatly increased below 202nm and varies with the instrument sophistication.

The observed absorption at 203nm by phosphatidylcholine results primarily from the double bonds in the fatty acid moiety of this lipid. This was demonstrated in the following experiment. Synthetic phosphatidylcholines of known fatty acid composition and of varying degree of unsaturation obtained from the commercial sources were first purified by h.p.l.c. The major peak of each of the synthetic phosphatidylcholines was collected. The amount of the phospholipid in the collected peak was determined by the P analyses (Dittmer & Wells, 1969). Known amounts of the purified standards were reinjected. The response in terms of peak area per mol of phospholipid was determined. The triangulation method was used to determine mean absorbance and concentration, and the apparent molecular extinction coefficient at 203nm was calculated. Table 1 indicates that the observed absorption at 203nm for the phosphatidylcholines was primarily due to isolated double bonds, and the response measured varied with the degree of unsaturation in the phosphatidylcholines.

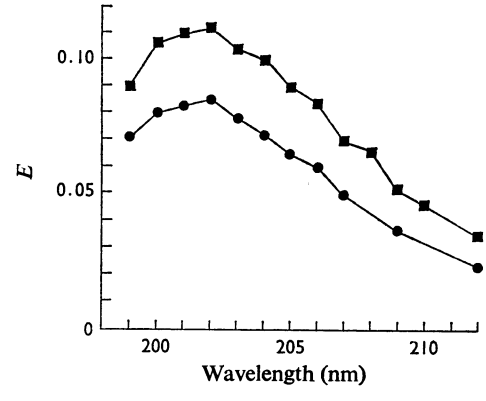


Fig. 3. *Apparent absorption spectra of phosphatidylcholine and sphingomyelin*

Phosphatidylcholine (10nmol; ■) and sphingomyelin (7.4nmol; ●) standards were injected. The ultraviolet response in terms of absorbance units (peak height) at different wavelengths is shown. Other h.p.l.c. conditions were the same as those described in Fig. 1. The points represent the average of two independent observations.

Table 1. *Apparent extinction coefficients* ($\varepsilon$) *of synthetic phosphatidylcholines*

Known amounts of purified synthetic phosphatidylcholines were chromatographed according to the h.p.l.c. conditions described in Fig. 1. The observed peaks were triangulated. The elution volume of the peak was measured as the base of the triangle from known flow rates. The mean absorbance of the peak was measured as one-half the peak height in *E* units. The apparent $\varepsilon$ were calculated from these data. The peak area was measured arbitarily (in mg) by the cut-and-weigh method. The data represent the average of three experiments.

| Fatty acids of phosphatidylcholine | Ultraviolet response [peak area (mg)/$\mu$mol of phospholipid] | Apparent $\varepsilon_{203}$ ($M^{-1} \cdot cm^{-1}$) |
|---|---|---|
| Di $C_{18}$ | 0.21 | $0.02 \times 10^4$ |
| Di $C_{18:1}$ | 4.43 | $0.43 \times 10^4$ |
| Di $C_{18:2}$ | 13.44 | $1.29 \times 10^4$ |
| Di $C_{18:3}$ | 19.16 | $1.89 \times 10^4$ |
| Di $C_{20:4}$ | 34.6 | $3.24 \times 10^4$ |

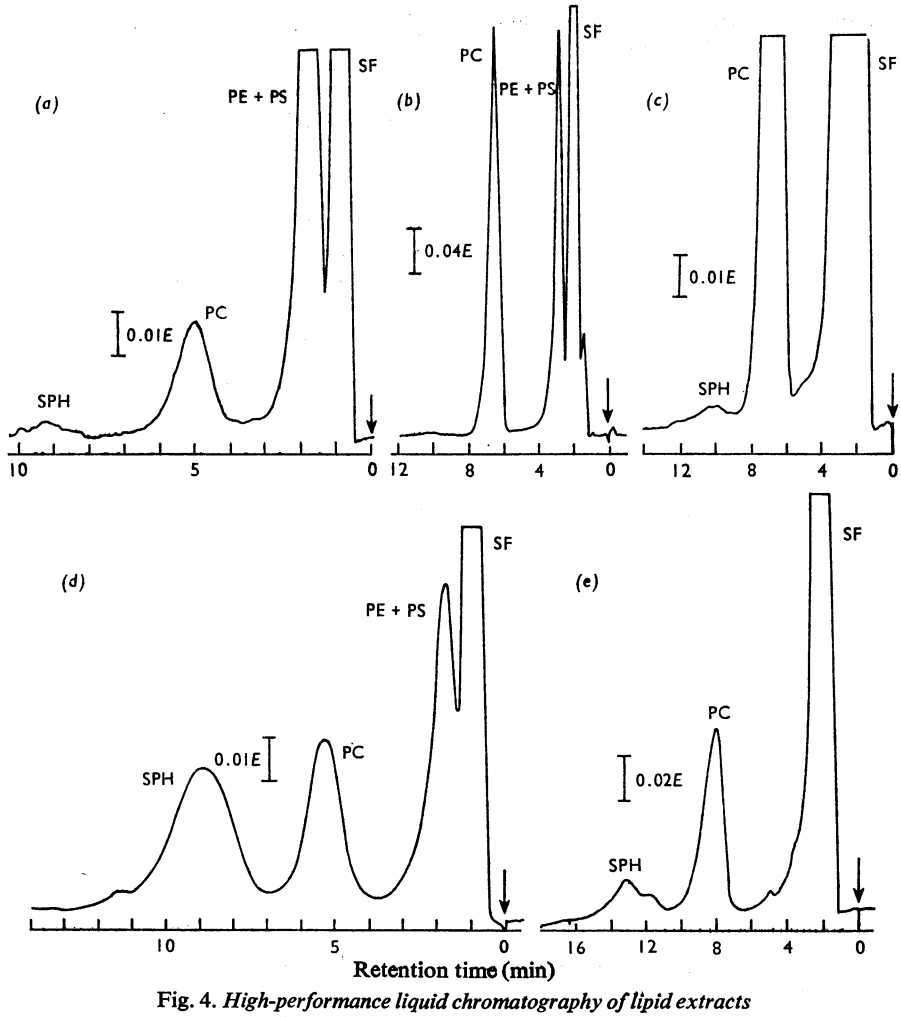


Fig. 4. *High-performance liquid chromatography of lipid extracts*

Extracts were from: (*a*) rat brain, 1.0 mg wet wt.; (*b*) and (*c*) rat liver, 0.7 and 1.1 mg wet wt. respectively; (*d*) human erythrocytes from 25 $\mu$l of whole blood; (*e*) human amniotic fluid, 1.8 ml. The h.p.l.c. conditions were the same as described in Fig. 1 for rat brain (*a*) and human erythrocyte (*d*) extracts. For extracts of liver (*b* and *c*) and amniotic fluid (*e*) the solvent used was acetonitrile/methanol/water (67:20:13, by vol., and 35:9:6, by vol., respectively). The arrow represents the point of injection. SF, solvent front; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SPH, sphingomyelin.

*Analysis of tissue extracts*

Lipid extracts from rat brain, rat liver, human blood and amniotic fluid were dissolved in ethanol and injected for h.p.l.c. analysis (Fig. 4). Phosphatidylcholine and sphingomyelin peaks from brain, blood and amniotic fluid could be readily detected. The response due to sphingomyelin in rat liver and brain extracts was low. However, by injecting the extract at higher concentration or by changing the sensitivity, the response due to sphingomyelin in these extracts could be clearly seen (e.g. Fig. 4*c*). The phosphatidylcholine and sphingomyelin peaks from blood and brain lipid extracts were collected and shown to be chromatographically pure by t.l.c. (Jungalwala *et al.*, 1975). These results indicated that the h.p.l.c. analysis of phosphatidylcholine and sphingomyelin in these tissue extracts could be made without interference from other detectable compounds in the extract. With the chromatographic system used for the analysis of these two phospholipids, phosphatidylethanolamine, phosphatidylserine, cerebrosides and sulphatides also gave response, but they were eluted near the solvent front. It was also possible to analyse phosphatidylcholine and sphingomyelin in the tissue extracts which had been subjected to biphenylamide formation for the analysis of the phosphatidylserine and phosphatidylethanolamine by the h.p.l.c. method (Jungalwala *et al.*, 1975). No apparent loss of phosphatidylcholine or sphingomyelin, or interference by other material, was observed. The quantities of phosphatidylcholine and sphingomyelin in the natural extracts as determined by the h.p.l.c. method with bovine brain phospholipid standards did not agree well with those determined by t.l.c. separation and phosphorus analysis. This apparently is due to differences in the fatty acid composition of the phospholipid standards used (bovine brain phospholipids) and in the fatty acid composition of the lipid extract tested with the h.p.l.c. method. However, quantitative results are obtained by the h.p.l.c. method if the phosphatidylcholine and sphingomyelin standards employed have the same fatty acid composition as the samples tested.

## Discussion

The utilization of h.p.l.c. with a solvent system transparent to ultraviolet light and detection in the region of 200nm provides a generally applicable method for the non-destructive analysis of unsaturated lipids with a high degree of sensitivity. The sensitivity of the method is dependent upon the apparent molecular extinction coefficients ($\varepsilon$) of the molecules analysed. In the case of lipids, the presence of double bonds is primarily responsible for the high degree of absorption observed. The $\varepsilon$ of an isolated double bond varies between 10000 and 15000 at wavelength maximum 179–196nm (Scott, 1964). Other chromophores in lipids, such as ester carbonyl ($\varepsilon_{201} \approx 50$), contribute little towards the observed absorption. In the case of phosphatidylcholines (Table 1) it is shown that the apparent $\varepsilon$ increased in proportion to the number of double bonds in the molecule. Murawski *et al.* (1974) have described a method for the detection and quantitative determination of serum and tissue lipids by measuring their ultraviolet absorption in the range of 196–210nm, after t.l.c. Direct quantification of phosphatidylcholine and sphingomyelin by h.p.l.c. and ultraviolet absorption can be obtained if the apparent $\varepsilon$ of the material analysed is established. A simple way to determine the apparent $\varepsilon$ is by performing h.p.l.c. of a representative sample, and measuring the response and the amount of phosphorus in the eluted peaks. Subsequent quantitative analysis can then be made directly from the ultraviolet response. However, if it is suspected that the degree of unsaturation in the lipids to be analysed could vary, peaks could be collected and quantified by independent micromethods. In the case of phospholipids, micromethods such as the fluorescent method (Schiefer & Neuhoff, 1971) or the phosphorus micro-assay (Dittmer & Wells, 1969; Hess & Derr, 1975) could be used.

As shown in Fig. 4 the analyses of phosphatidylcholine and sphingomyelin by h.p.l.c. were made on the tissue lipid extracts without interference from other phospholipids or ultraviolet-absorbing material. The low response observed for sphingomyelin in the case of rat brain and liver is consistent with the reported content of sphingomyelin and the unsaturation in fatty acid moiety of sphingomyelin in relation to phosphatidylcholine (White, 1973; Abe & Norton, 1974).

The h.p.l.c. method described here for the analysis of phosphatidylcholine and sphingomyelin is complementary to the h.p.l.c. method described by Jungalwala *et al.* (1975) for the analysis of ethanolamine-containing and serine-containing phosphoglycerides. The quantitative range of derivative formation and h.p.l.c. analysis of the biphenylamides of the aminophospholipids was shown to be 10–500nmol. However, the lower limit of detection by h.p.l.c. of the phospholipid derivatives was about 10–13pmol or 0.3–0.4ng of P. The sensitivity of the present method for the analysis of phosphatidylcholine and sphingomyelin is dependent mainly upon the degree of unsaturation. Approx. 1nmol of phosphatidylcholine, containing at least one double bond per molecule, can be detected. The advantage of these h.p.l.c. methods, besides their sensitivity and speed, is that they are non-destructive. One can recover the injected sample, and then perform subsequent chemical determinations or analysis for radioactivity or both.

Recently we have initiated studies on the analysis and detection at 203nm of all the complex lipids in the tissue extracts by gradient-elution h.p.l.c. It has been possible so far to resolve the lipids of brain into four major fractions by this method. Cholesterol, cerebrosides and sulphatides were eluted with the solvent front. Phosphatidylserine and phosphatidylethanolamine were then eluted together, followed by phosphatidylcholine, sphingomyelin and lysophosphatidylcholine.

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