Sensitive Analysis of Ethanolamine- and Serine-Containing Phosphoglycerides by High-Performance Liquid Chromatography

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A highly sensitive method for the separation and quantitative measurement of phospholipids containing primary amino groups, such as phosphatidylethanolamine, phosphatidylserine and lysophosphatidylethanolamine, is described. The method involves a simple and quantitative derivative formation of the phospholipids containing amino groups to their u.v.-absorbing biphenylcarbonyl derivatives. These have molar extinction coefficients of about 23 000 at 268 nm. The phospholipid derivatives are then separated and non-destructively determined by high-performance liquid chromatography. The amino phospholipids containing vinyl ether bonds (plasmalogens) can be determined separately from the diacyl- and alkylacyl-amino phospholipids. The lower limit of detection by high-performance liquid-chromatographic analysis of the phospholipid derivatives is about 10–13 pmol or 0.3–0.4 ng of phospholipid P. The quantitative range of derivative formation and analysis by high-performance liquid chromatography of the phospholipids containing amino groups was shown to be 10–500 nmol. The method was shown to be applicable to the analysis of phospholipids containing amino groups in tissue samples.

The speed and efficiency of liquid-chromatographic analysis has been considerably increased by the development of high-pressure pumping systems, controlled-surface-porosity column packings and minimum-flow detectors (Snyder & Kirkland, 1974). However, successful application of modern high-performance liquid chromatography has been primarily limited to materials that absorb u.v. light, because u.v. monitors (254 and 280 nm) are the most sensitive and practical detectors available. In cases where the compounds to be analysed by high-performance liquid chromatography do not absorb u.v. light, their derivatives have been made and subsequently analysed by high-performance liquid chromatography. Thus Evans & McCluer (1972) and Sugita *et al.* (1974) have analysed cerebrosides and ceramides as their benzoylated derivatives by high-performance liquid chromatography. Phospholipids do not absorb u.v. light in the region 254–280 nm. However, the primary amino group present in certain phospholipids, such as ethanolamine- and serine-containing phospholipids, can be easily converted into their *N*-acyl derivatives. In this report, we describe a highly sensitive method for the separation and determination of phospholipids that contain amino groups as their biphenylcarbonyl derivatives by high-performance liquid chromatography. Part of this work has been previously presented (Jungalwala *et al.*, 1974).

Experimental

Materials and methods

4-Biphenylcarbonyl chloride was obtained from Aldrich Chemical Co. Milwaukee, Wis., U.S.A. Fluorescamine, 4-phenylspiro[furan-2(3H),1'-phthalan]-3,3-dione was from Hoffmann-La Roche, Nutley, N.J., U.S.A. Synthetic *L*- α -distearoyl, *L*- α -dipalmitoyl and *L*- α -dioleoyl phosphatidylethanolamines as well as naturally occurring phospholipids were obtained from Supelco, Bellefonte, Pa., U.S.A. Their purity was checked by t.l.c. (Horrocks, 1968; Eichberg *et al.*, 1973). Radioactive [^{14}C]phosphatidylethanolamine and [^{14}C]phosphatidylserine were prepared from rat brains by the intracerebral injection into six 21-day-old rats of 25 μCi of [$2\text{-}^{14}\text{C}$]ethanolamine (27 mCi/mmol; Amersham Searle, Arlington Heights, Ill., U.S.A.) or of 50 μCi of *L*-[$U\text{-}^{14}\text{C}$]serine (120 mCi/mmol; ICN Pharmaceuticals, Cleveland, Ohio, U.S.A.) per animal (Jungalwala & Dawson, 1971). The lipids were extracted from the rat brains with 20 vol. of chloroform-methanol (2:1, v/v) as described previously (Jungalwala & Dawson, 1971). The extracts were washed three times with 0.2 vol. of a mixture containing chloroform-methanol-water-10 mM-ethanolamine or -serine (3:45:46:1, by vol.) and three times with the same mixture but without added ethanolamine or serine. The washed lipid extracts (about

400 mg of lipid) were applied to a 30 g Unisil (Clarkson Chemicals, Williamsport, Pa., U.S.A.) column (1.5 cm × 15 cm) by the procedure of Vance & Sweeley (1967). After removal of the non-polar-lipid fraction and glycolipids, the methanol fraction was collected which contained most of the phospholipids. This fraction was decreased in volume and applied to a neutral alumina column as described by Luthra & Sheltawy (1972). The radioactive ethanolamine- or serine-containing phosphoglycerides were eluted as a single peak and were shown to be free from other phospholipids by t.l.c. (Horrocks, 1968; Eichberg *et al.*, 1973), radioautography and by scanning the t.l.c. plates with a Berthold radioscanner.

All solvents were of reagent grade.

Preparation of derivatives

The phospholipids containing amino groups were treated with 4-biphenylcarbonylchloride in two different ways.

Procedure (a). The individual standard phospholipids (about 10–100 nmol) were dried over P_2O_5 in a vacuum desiccator. Then 0.1–0.25 ml of 4-biphenylcarbonyl chloride (0.2%, w/v) in acetonitrile was added to the dried phospholipids, followed by 2–5 μ l of triethylamine. The mixture was shaken vigorously at 37°C for 30 min.

Procedure (b). Alternatively, to the dried phospholipids (about 10–100 nmol) 20–50 μ l of 1% (w/v) solution of 4-biphenylcarbonyl chloride in tetrahydrofuran was added, followed by 40–100 μ l of aq. 50% (w/v) sodium acetate. The mixture was shaken vigorously at 22°C for 60 min.

The reaction products from either procedure (a) or (b) were purified as follows: 1 vol. of chloroform-methanol mixture (2:1, v/v) was equilibrated with 0.2 vol. of water and the phases were allowed to separate. The upper phase was discarded and 5 ml of the lower phase was added to the reaction mixture. The excess of reagent was removed by washing the solution twice with 1 ml of methanol-0.9% NaCl-chloroform-15 M- NH_3 (96:92:2:3, by vol.). The lower phase was rewashed three times with 1 ml of methanol-0.9% NaCl-chloroform (48:47:1 by vol.). The lower phase was evaporated to dryness under a stream of N_2 . The residue was then dissolved in about 50–200 μ l of chloroform-methanol (1:1, v/v). From this point on, all samples in screw-cap tubes (Reactivials; Pierce Chemical Co., Rockford, Ill., U.S.A.) were kept at 0–4°C to minimize evaporation of the solvent.

Tissue lipid extracts

The microsomal fraction and myelin were prepared from 30-day-old rat brains by the method of Jungalwala (1974). Adult rat brain, liver or the subcellular

fractions were homogenized in 20 vol. of chloroform-methanol (2:1, v/v) and filtered. The residue was re-extracted with 20 vol. of the same solvent. The combined extracts were washed once with 0.2 vol. of 0.9% NaCl and three times with 0.2 vol. of theoretical upper-phase solvent consisting of methanol-0.9% NaCl-chloroform (48:47:7, by vol.). Total lipid P in the extracts was determined by the method of Bartlett (1959). The amounts of phosphatidylethanolamine and phosphatidylserine in the extracts were also determined. The lipids in the extracts were first separated by a two-dimensional t.l.c. procedure on silica G (Analtech, Newark, Del., U.S.A.) plates (Horrocks, 1968) or on silica gel HR plates (Analtech) (Eichberg *et al.*, 1973). The phosphatidylethanolamine and phosphatidylserine spots from the plates were recovered and extracted, and the amounts of lipid P in the extract were determined. These values were used for comparison with those obtained by the high-performance liquid-chromatography method. Portions of the tissue extracts were dried under N_2 and used directly for derivative formation as described above.

Hydrolysis of ethanolamine plasmalogens

For the analysis of ethanolamine plasmalogens present in the tissue extracts, a portion of the derived extract was dried under N_2 in Reactivials (1 ml size). The open vial was then inverted and held over an open bottle of conc. HCl for 10 min. The HCl fumes remaining in the vial were removed by flushing with N_2 for a few minutes. The vial was cooled in an ice bath and chloroform-methanol (1:1, v/v) was added in an amount identical with that of the original sample. The biphenylcarbonyl derivative of lysophosphatidylethanolamine thus formed was then measured by high-performance liquid chromatography.

Liquid chromatography

High-performance liquid-chromatographic analysis was performed with a Perkin-Elmer model 1220 high-pressure liquid chromatograph equipped with a Laboratory Data Control (Riviera Beach, Fla., U.S.A.) model 1285 u.v. monitor (280 nm). The u.v. monitor has an 8 μ l flow cell. The chromatographic column was a 20 cm × 2.1 mm (internal diam.) pre-packed stainless-steel 'MicroPak' SI-10 column (Varian, Palo Alto, Calif., U.S.A.), average particle diameter of silica gel 10 μ m. Since the polarity of the phosphatidylethanolamine, lysophosphatidylethanolamine and phosphatidylserine derivatives is quite different, solvents of different polarity were used to elute them from the column. For the analysis of the phosphatidylethanolamine derivative, the solvent used was dichloromethane-methanol-15 M- NH_3 .

(92:8:1, by vol.), and the lysophosphatidylethanolamine and phosphatidylserine derivatives the same solvents in the proportions 80:15:3 (by vol.) were used. Sometimes, the lysophosphatidylethanolamine derivative was analysed with the above solvent mixture in the proportions 80:12:1 (by vol.). Under this condition, the phosphatidylserine derivative is not eluted from the column. The samples (1–5 nmol) dissolved in chloroform–methanol (1:1, v/v) were injected in a volume of 2–10 μ l. The elution was performed at a flow rate of 1 ml/min. The column inlet pressure was approximately 500 lb/in². The attenuator on the u.v. monitor was normally set at 0.04 absorbance unit full scale. However, for very sensitive analysis (pmol lipid range), the attenuator on the u.v. monitor and the recorder were set so as to read 0.004 absorbance unit full scale. For integration of the peak areas the recorder was coupled to a Hewlett–Packard 3370B integrator. Alternatively, the peak areas were measured by the cut-and-weigh method.

Results

Comments on derivative formation

Either procedure (a) or (b) described in the Experimental section is suitable for preparing derivatives of phospholipids containing amino groups. However, method (b) is preferred, since with this method the formation of side products, mainly the anhydride of biphenylcarboxylic acid, appeared to be less. The amount of biphenylcarbonyl chloride used for derivative formation is dependent on the amount of phospholipids containing amino groups in a mixture. With either procedure, a 20 mol excess of the reagent over the amino phospholipids was more than sufficient for derivative formation, and a 75 mol excess of reagent did not appear to interfere with the analysis. However, greater than 75 mol excess of the reagent forms large amounts of the anhydride, which can interfere with the liquid-chromatographic analysis. For unknown amounts of phospholipids containing amino groups, e.g. in tissue extracts, a

Fig. 1. Radioactivity scan of a t.l.c. plate spotted with phosphatidylethanolamine (PE) and the biphenylcarbonyl derivative of the same amount of phosphatidylethanolamine (PE derivative)

The t.l.c. plate (silica gel GF) was developed with chloroform–methanol–acetic acid–water (100:25:8:4, by vol.). Abbreviations: OR, origin; S.F., solvent front.

portion was first used to determine the total phospholipid P, and it was assumed that 40–50% of the phospholipids in the sample were phospholipids containing amino groups.

Identification of products

Fig. 1 shows a radioactivity scan of a t.l.c. plate spotted with radioactive phosphatidylethanolamine (about 5500 d.p.m.; 8 μ g of P) and the same amount of radioactive phosphatidylethanolamine that had been subjected to derivative formation. It is evident (Fig. 1) that only a single radioactive product is formed by derivative formation of phosphatidylethanolamine. All the radioactivity in the reaction mixture spotted on the t.l.c. plate was recovered in the spot corresponding to the biphenyl derivative. Fig. 2 shows a t.l.c. radioactivity scan of phosphatidylserine (about 3300 d.p.m.; 11 μ g of P) and its biphenylcarbonyl derivative.

The phospholipid derivatives were characterized by t.l.c. procedures. On a silica gel GF plate (E. Merck, Elmsford, N.Y., U.S.A.) developed with chloroform–methanol–acetic acid–water (100:25:6:2,

by vol.), the R_f values were: phosphatidylethanolamine, 0.24; its biphenylcarbonyl derivative, 0.7; phosphatidylserine, 0.09; its biphenylcarbonyl derivative, 0.27; lysophosphatidylethanolamine, 0.07; its biphenyl carbonyl derivative, 0.33. The anhydride of the biphenylcarbonyl acid had an R_f value of 0.96. When the t.l.c. plate was viewed under short-wavelength u.v. light (Chromatovue CC-20 lamp) the biphenylcarbonyl derivatives appeared as quenched spots on a fluorescent background. The lanes containing lipid derivatives on the t.l.c. plate did not give a positive ninhydrin colour reaction nor fluorescamine reaction (Felix & Jimenez, 1974). Single fluorescent spots in each lane appeared when the t.l.c. plate was sprayed with 8-anilino-1-naphthalenesulphonic acid spray (Gitler, 1972). Phosphate-positive spots, corresponding to the fluorescent spots from the 8-anilino-1-naphthalenesulphonic acid spray, were seen when the same plate was sprayed with a phosphate spray (Vaskovsky & Kostetsky, 1968). The recovery of the phospholipids after derivative formation, on the basis of radioactivity and P determination, varied between 95 and 102%.

Fig. 2. Radioactivity scan of a t.l.c. plate spotted with phosphatidylserine (PS) and its biphenylcarbonyl derivative (PS derivative)

The t.l.c. plate (silica gel GF) was developed with chloroform–methanol–15M-NH₃ (10:5:1, by vol.). Abbreviations: OR, origin; S.F., solvent front.

Fig. 3. High-performance liquid-chromatographic analysis of biphenylcarbonyl derivatives of *L*- α -distearoyl phosphatidylethanolamine (a) and *L*- α -dioleoyl phosphatidylethanolamine (b)

The solvent was dichloromethane-methanol-15M-NH₃ (92:8:1, by vol.). The recorder response was set at 0.04 absorbance unit full scale at 280 nm. The amounts injected were equivalent to 0.95 nmol of distearoyl phosphatidylethanolamine (29.4 ng of P) and 0.97 nmol of dioleoyl phosphatidylethanolamine (30.0 ng of P). $\downarrow$ represents point of injection. Abbreviation: S.F., solvent front; PE, phosphatidylethanolamine.

The biphenylcarbonyl derivatives of phosphatidylethanolamine and phosphatidylserine were purified by the high-performance liquid-chromatography method. The molar extinction coefficients of the phosphatidylethanolamine and phosphatidylserine in methanol were 22 500 and 23 800 respectively at $\lambda_{\max}$, 268 nm, and 20 000 and 20 300 respectively at 280 nm, where the phospholipids are analysed by high-performance liquid chromatography. The molar extinction coefficient of biphenylcarbonyl chloride in methanol at $\lambda_{\max}$, 270 nm was 21 000. These observations suggested that only one H⁺ of the NH₂ group of the phospholipids was displaced by a biphenylcarbonyl group.

Separation by high-performance liquid chromatography

The high-performance liquid-chromatographic analysis of the 4-biphenylcarbonyl derivative of synthetic *L*- α -distearoyl phosphatidylethanolamine and *L*- α -dioleoyl phosphatidylethanolamine is shown in Fig. 3. The retention times of these two compounds

were slightly different, 5.3 and 4.9 min respectively (K' , number of column volumes of solvent required to elute the chromatographed substance = 6.5 and 6.9), presumably owing to differences in the fatty acid moieties of the phospholipids. *L*- α -Dipalmitoyl phosphatidylethanolamine had the same retention time as that of the stearoyl form. The lysophosphatidylethanolamine derivative was eluted at a retention time of about 14–18 min ($K' = 20$) under the conditions described in Fig. 3. The biphenylcarbonyl derivative of phosphatidylserine was not eluted from the column under this condition. The anhydride of biphenylcarbonyl acid eluted with the solvent front.

Fig. 4 shows the high-performance liquid chromatographic analysis of the biphenylcarbonyl derivatives of lysophosphatidylethanolamine (bovine; Supelco) and phosphatidylserine (bovine). The phosphatidylserine derivative peak under these conditions (Fig. 4b) shows a shoulder, probably because of the different fatty acids present in the sample. We have been able to resolve the shoulder into a separate peak under slightly different chrom-

Fig. 4. High-performance liquid-chromatographic analysis of biphenylcarbonyl derivatives of (a) lysophosphatidylethanolamine (LysoPE) and (b) phosphatidylserine (PS)

The solvent was dichloromethane-methanol-15M-NH₃ (80:15:3, by vol.). The amounts injected were equivalent to 0.87 nmol of lysophosphatidylethanolamine (26.8 ng of P) and 3.2 nmol of phosphatidylserine (98.3 ng of P). Abbreviation: S.F., solvent front.

atographic conditions. The phosphatidylethanolamine derivative was eluted with the solvent front under the conditions described in Fig. 4. Better resolution of the lysophosphatidylethanolamine derivative from the solvent front was achieved with a slightly less polar solvent mixture of dichloromethane-methanol-15M-NH₃ (80:12:1, by vol.). The phosphatidylserine derivative was not eluted from the column under these conditions. On the other hand, higher sensitivity was achieved for phosphatidylserine analysis with a slightly more polar solvent, which eluted the phosphatidylserine derivative as a sharper peak.

Quantitative measurement of phosphatidylethanolamine, phosphatidylserine and lysophosphatidylethanolamine

The performance of the column and the detection system on injection of increasing amounts (from a

single sample) of the derivatives of phosphatidylethanolamine and phosphatidylserine was determined. The response was linear when 10 pmol to 20 nmol of the derivatives were injected. The percentage relative standard deviation of the mean for repeated injections of the same sample (1 nmol of phospholipid per injection) was 0.5%. The retention times were independent of the sample quantities (1–20 nmol). The lower limit of detection and analysis of the phospholipid derivatives was approx. 10–13 pmol (0.3–0.4 ng of P).

To test the quantitative range of the entire procedure (derivative formation and high-performance liquid chromatography) for the analysis of phosphatidylethanolamine and phosphatidylserine, various amounts of the lipid samples (10–500 nmol) were treated with biphenylcarbonyl chloride. The samples of lipid derivatives were then dissolved in chloroform-methanol (1:1, v/v) so that the concentration of the original lipid was about 1 nmol/5 μ l of

the solvent and 5 μ l (1 nmol) portions of the samples were injected on the column for high-performance liquid-chromatographic analysis. The observed response, in terms of peak area, was then calculated for the total amount reacted. Fig. 5 shows that the calculated response was linear for the results of the lipids that had reacted. Similar amounts were obtained for lysophosphatidylethanolamine. Though the minimum amount of the phospholipids that had reacted as described here was about 10 nmol, it should be possible to make derivatives of and analyse much smaller amounts (pmol range) by using micro-techniques and proportionately decreasing the amount of reagent.

Analysis of phosphatidylethanolamine, ethanolamine plasmalogens and phosphatidylserine in tissue samples

Total lipid extracts (containing about 10–50 μ g of total phospholipid P) from adult rat brain or liver as well as those of microsomal and myelin fractions of 30-day-old rat were converted into biphenylcarbonyl derivatives and injected. Fig. 6(a) shows the high-performance liquid-chromatographic analysis

Fig. 5. Quantitative analysis of phosphatidylethanolamine and phosphatidylserine by derivative formation and high-performance liquid chromatography

The amounts of phospholipids indicated in the figure were converted into derivatives as described in the Experimental section. A small amount, about one nmol, of the total sample was injected for high-performance liquid-chromatographic analysis. The response in terms of peak area per total sample was calculated. ●, Phosphatidylethanolamine; ▲, phosphatidylserine.

Fig. 6. High-performance liquid-chromatographic analysis of biphenylcarbonyl derivatives of the lipid extract of myelin before (a) and after (b) exposure to HCl

A lipid extract of myelin containing approx. 9 μ g of total phospholipid P was treated with biphenylcarbonyl chloride as described in the Experimental section. The lipid extract, which was converted into derivatives, was then dissolved in 200 μ l of chloroform-methanol (1:1, v/v) and a 5 μ l portion was injected into the high-performance liquid-chromatography column. (a) Phosphatidylethanolamine derivative including ethanolamine plasmalogen; (b) a 50 μ l portion was dried and exposed to HCl fumes, redissolved in 50 μ l of the same solvent and a 5 μ l sample injected. Diacyl- and alkylacyl-phosphatidylethanolamine derivatives are eluted at a retention time of about 4 min. Lysophosphatidylethanolamine formed is eluted with a retention time of 14–20 min. The solvent was dichloromethane-methanol-15M-NH₃ (92:8:1, by vol.). The vertical marks of the curves are due to signals from the integrator. Abbreviations: PE, phosphatidylethanolamine; S.F., solvent front.

Fig. 7. High-performance liquid-chromatographic analysis of biphenylcarbonyl derivatives of myelin lipid extract

The same lipid extract, which was converted into derivatives and described in Fig. 6, was injected with the solvent system dichloromethane-methanol-15M-NH₃ (80:15:3, by vol.). (a) 2 μ l and (c) 10 μ l of myelin lipid derivative extract before exposure to HCl. (b) 2 μ l sample of the same lipid extract after exposure to HCl. Abbreviations: PS, phosphatidylserine; LysoPE, lysophosphatidylethanolamine; S.F., solvent front.

Table 1. Phospholipid composition of tissue extracts

The values are expressed as percentages of total phospholipid P. The high-performance liquid-chromatography results are averages ($\pm$ S.D.) of eight experiments. The t.l.c. results are averages of three experiments. T.l.c. was performed as described in the text. Ethanolamine plasmalogens were determined by the decrease in the amount of phosphatidylethanolamine and the increase in the amount of lysophosphatidylethanolamine after exposure of the lipid extract to HCl fumes. References: (a) Wuthier (1966); (b) data calculated from Norton & Poduslo (1973); (c) data calculated from Cuzner *et al.* (1965); (d) Herschkowitz *et al.* (1968).

Phospholipid	Liquid chromatography	T.l.c.	As reported in literature
Liver (rat)			
Phosphatidylethanolamine	22.8 $\pm$ 1.9	23.5	25.2 (a)
Phosphatidylserine	2.4 $\pm$ 0.4	2.4	3.2 (a)
Brain (rat)			
Phosphatidylethanolamine	41.6 $\pm$ 2.6	38.1	36.4 (a), 34.8 (b), 41.1 (c)
Ethanolamine plasmalogens	22.9 $\pm$ 1.3	—	19.9 (b), 22.3 (c)
Phosphatidylserine	11.7 $\pm$ 1.6	13.2	11.8 (a), 12.5 (b), 11.6 (c)
Rat brain microsomal fraction			
Phosphatidylethanolamine	32.6 $\pm$ 1.8	28.7	33.6 (c)
Ethanolamine plasmalogens	17.1 $\pm$ 1.0	—	14.0 (c)
Phosphatidylserine	10.9 $\pm$ 1.1	11.2	10.7 (c)
Rat brain myelin			
Phosphatidylethanolamine	41.5 $\pm$ 2.7	41.9	37.8 (b), 45.0 (c), 39.3 (d)
Ethanolamine plasmalogens	33.9 $\pm$ 1.2	—	30.0 (b), 35.4 (c)
Phosphatidylserine	12.6 $\pm$ 0.6	—	16.0 (b), 15.5 (c), 14.6 (d)

of the phosphatidylethanolamine derivative of a myelin lipid extract. After exposure of the same sample of the lipid derivative extract to HCl, the amount of ethanolamine phosphoglycerides decreased to about 22% of the original (Fig. 6b). This was due to the conversion of the ethanolamine plasmalogen derivative into a lysophosphatidylethanolamine derivative, which was eluted from the column with a retention time of 14–20 min. The original extract which was treated and injected under the conditions for the high-performance liquid-chromatographic analysis of lysophosphatidylethanolamine and phosphatidylserine, showed the presence of only a minute amount of lysophosphatidylethanolamine (Fig. 7a). However, when the same sample of the derivative extract exposed to HCl was injected a sharp peak corresponding to the lysophosphatidylethanolamine derivative appeared (Fig. 7b). The phosphatidylserine-derivative peak from the myelin extract was not significantly decreased after exposure to HCl, indicating the presence of little serine plasmalogen. No attempts were made to identify lysophosphatidylserine derivative by high-performance liquid chromatography. When larger amounts of the myelin lipid derivative extract (five times the amount shown in Fig. 7a) was injected, small downslope shoulders on the solvent front with retention times similar to that of lysophosphatidylethanolamine derivative were obtained (Fig. 7c). However, their identity has not been confirmed. The phospholipid composition of the extracts was determined by measuring the total phospholipid P in the extract and then determining the quantity of individual phospholipids by derivative formation and high-performance liquid-chromatographic analysis along with known amounts of standards. The detector response per amount of phospholipid P of the standards was compared with the response, obtained under the same conditions, from test samples. The quantitative high-performance liquid-chromatographic analysis of the amino phospholipids in the rat brain and liver extracts as well as that of rat brain myelin and microsomal fractions were compared with the results obtained by the t.l.c. method and with values reported by other investigators (Table 1). Good agreement between the two methods and the literature values is evident.

Discussion

The existing methods for the separation and determination of microgram quantities of phospholipids require t.l.c. or alkaline and acid hydrolysis followed by paper chromatography and high-voltage ionophoresis (Spanner, 1973). The separated individual phospholipids are then digested and the P_i is determined colorimetrically. These methods are time-consuming and not sensitive enough for the

analysis of minute amounts of tissues or membranous materials. A fluorimetric assay for the determination of phospholipids has been published (Schiefer & Neuhoﬀ, 1971). However, in this method, the separation of the phospholipids on t.l.c. plates and the elution of the individual phospholipids from the plates is a prerequisite to the quantitative determination with the fluorescent reagent Rhodamine 6G. Again, special efforts are needed to eliminate quenching effects. The high-performance liquid-chromatographic method described in the present paper for the analysis of phosphoglycerides containing ethanolamine and serine has about the same sensitivity as that of the fluorimetric method. In the procedure presented here, the analysis of the phosphoglycerides containing ethanolamine and serine in a mixture is not possible with a single injection owing to the difference in polarity of the solvents needed for elution. One could devise a gradient-elution procedure; however, the re-equilibration of absorbent activity necessary for subsequent injection may require considerable time, and thus may not be as convenient as isocratic analysis. In practice, we have found that one can analyse a series of samples for phosphatidylethanolamine and subsequently analyse the samples for lysophosphatidylethanolamine and phosphatidylserine with the more-polar solvent system. The advantage of the high-performance liquid-chromatographic method, besides its sensitivity, is that it is non-destructive and one can recover the injected sample. Recently, one of us has used the method to analyse the specific radioactivity of ^{14}C -labelled phosphatidylethanolamine and phosphatidylserine in purified myelin and microsomal fractions of brain from young animals and in tissue slices (F. B. Jungalwala, unpublished work). The method described in this paper is also applicable for the highly sensitive analysis of sphingolipids containing amino groups, such as sphingosine, psychosine and sphingosylphosphorylcholine (F. B. Jungalwala, unpublished work).

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References

- Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
- Cuzner, M. L., Davison, A. N. & Gregson, N. A. (1965) *J. Neurochem.* **12**, 469–481
- Eichberg, J., Shein, H. M., Schwartz, M. & Hauser, G. (1973) *J. Biol. Chem.* **248**, 3615–3622
- Evans, J. E. & McCluer, R. H. (1972) *Biochim. Biophys. Acta* **270**, 565–569
- Felix, A. M. & Jiminez, M. H. (1974) *J. Chromatogr.* **89**, 361–364
- Gitler, C. (1972) *Anal. Biochem.* **50**, 324–325

- Herschowitz, N., McKhann, G. M. & Shooter, E. M. (1968) *J. Neurochem.* **15**, 161–168
- Horrocks, L. A. (1968) *J. Lipid Res.* **9**, 469–472
- Jungalwala, F. B. (1974) *J. Lipid Res.* **15**, 114–123
- Jungalwala, F. B. & Dawson, R. M. C. (1971) *Biochem. J.* **123**, 683–693
- Jungalwala, F. B., Evans, J. & McCluer, R. H. (1974) *Trans. Amer. Soc. Neurochem.* **5**, 63
- Luthra, M. G. & Sheltawy, A. (1972) *Biochem. J.* **126**, 251–253
- Norton, W. T. & Poduslo, S. E. (1973) *J. Neurochem.* **21**, 659–773
- Schiefer, H. G. & Neuhoﬀ, V. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 913–926
- Snyder, L. R. & Kirkland, J. J. (1974) *Introduction to Modern Liquid Chromatography*, Wiley-Interscience, New York
- Spanner, S. (1973) in *Form and Function of Phospholipids* (Ansell, G. B., Hawthorne, J. N. & Dawson, R. M. C., eds.), pp. 43–65, Elsevier, Amsterdam
- Sugita, M., Iwamori, M., Evans, J., McCluer, R. H., Dulaney, J. T. & Moser, H. W. (1974) *J. Lipid Res.* **15**, 223–226
- Vance, D. E. & Sweeley, C. C. (1967) *J. Lipid Res.* **8**, 621–630
- Vaskovsky, V. E. & Kostetsky, E. Y. (1968) *J. Lipid Res.* **9**, 396
- Wuthier, R. E. (1966) *J. Lipid Res.* **7**, 544–550