notes on methodology

Estimation of phosphatidylglycerol in fluids containing pulmonary surfactant

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Summary A simple, direct and non-destructive method for quantitative separation of phosphatidylglycerol from other phospholipids in pulmonary washings is described. Total lipid extracts from dog lung washings and phosphatidylglycerol standard were spotted quantitatively on chromatoplates and separated by one-dimensional thin-layer chromatography in chloroform–methanol–water 65:25:4 (v/v/v). Quantification was performed with Rhodamine 6G and fluorometry. Washings from eleven dogs contained (mean ± S.E.) 236 ± 25 μg phosphatidylglycerol per g of parenchymal wet lung tissue which accounted for 8.7% ± 2.3 of the total phospholipids. The procedure is especially useful for quantification of phosphatidylglycerol in microgram amounts.—**Cernansky, G., D-F. Liau, S. A. Hashim, and S. F. Ryan.** Estimation of phosphatidylglycerol in fluids containing pulmonary surfactant. *J. Lipid Res.* 1980. **21:** 1128–1131.

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Phosphatidylglycerol (PG) is the second most abundant phospholipid (the first being phosphatidylcholine) in pulmonary alveolar surfactant. Existing procedures (1–6) for isolation and estimation of PG require lengthy preparatory steps and two-dimensional or one-dimensional thin-layer chromatography (TLC) with two different solvent systems. This paper describes a simple procedure for separation of PG from other phospholipids of total lipid extracts utilizing one-dimensional TLC, one solvent system,

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Abbreviations: TLC, thin-layer chromatography; PL, phospholipid; PG, phosphatidylglycerol; PDME, phosphatidyldimethylethanolamine; PE, phosphatidylethanolamine; LBPA, lyso-bis-phosphatidic acid.

and commercially available chromatoplates. Our results are comparable to those obtained by other time-consuming methods.

MATERIAL AND METHODS

All phospholipid (PL) standards were purchased from Sigma Chemical Co., St. Louis, MO, and their purity was checked by thin-layer chromatography. All other chemicals were reagent grade.

Isolation of pulmonary surfactant material

Lung washings were obtained from healthy dog lungs, after degassing, using a total of 6 liters of Tris buffer containing 0.15 M sodium chloride, 0.003 M magnesium chloride, and 0.003 M calcium chloride (7). This exhaustive lavage of dog lung yields approximately 236 μg of PG per g of lung. The procedure is capable of separating and quantifying 1.5 μg of PG. Thus, the PG content of washings contributed by 6 mg of lung and represented by 0.38 ml of pooled washings can be detected. After centrifugation at 160 g for 10 min at 4°C, samples of the washings were lyophilized and extracted according to the method of Folch, Lees, and Sloane Stanley (8). The washed lipid extracts were concentrated on a flash evaporator, transferred into screw-top tubes and made to a volume of 20 ml with chloroform–methanol 2:1 (v/v). These lipid stock solutions were stored at −20°C. Total lipid phosphorus also was determined on samples of stock solutions according to a modified method of Beveridge and Johnson (9). We assumed that phospholipids contained an average of 4% phosphorus.

Thin-layer chromatography procedure; plates and application of samples

Chromatoplates, 20 × 20 cm, pre-coated with a 250 μm layer of silica gel 60 Kieselgel (Merck), were used; (Scientific Products, Div. of American Hospital Supply Corp., Edison, NJ). These plates were preferred over others because the adsorbent layer could withstand spraying to saturation without damage and because temperature and humidity had less adverse effects on these plates. The unknowns were spotted quantitatively (in triplicate) directly from their stock solutions with disposable micropipettes as single spots, approximately 2.3 cm apart and 2.5 cm from the bottom of the plates. To prevent evaporation of the stock solution during spotting, the tube was covered with a piece of Teflon tape and held in place with an o-ring. A small hole permitted the sampling. At least two spots of the PG standard (1 μg/μl) also were placed in the middle of the plate. Two μg of standard

were spotted and the unknowns were kept within the 1.5 to 4 μg range. A total of eight spots can be assayed on one plate. The plate was kept in the dark in a desiccator for at least 2 hr before development.

Solvent system and development of plates

The chromatographic chamber with dimensions of 10⅛″ × 11½″ × 3¾″ (Analabs, Inc., North Haven, CT) was lined on all sides with Whatman No. 1 filter paper wetted with the developing solvent chloroform–methanol–water 65:25:4 (v/v/v) (10) for at least 4 hr prior to use. The chamber was then saturated with fresh solvent for 1½ hr prior to development. The development took place in the dark at ambient temperature and humidity, usually for 1 hr. Thereafter, the plate was removed and air-dried completely in the dark.

Spraying

The dry, developed plate was placed almost horizontally in a 15″ × 15″ polyvinyl chloride spray box and sprayed (Desaga glass atomizer from Brinkham Instruments, Westbury, NY) to saturation with 0.05% Rhodamine 6G (National Aniline Division, Allied Chemical Corp., New York, NY) in 50% ethanol. A uniform spraying prevented gross irregularities in the baseline. The plate was again thoroughly air-dried in the dark before scanning.

Fluorometric scanning and quantification

A Turner Model 111 filter fluorometer equipped with a Camag-Turner Model 2 automatic thin-layer chromatogram scanner was used for measuring the fluorescence produced by the phospholipid spots. The ultraviolet light source was a 4-watt UV lamp with a maximum wave length emission at 360 nm (Turner #110-850). Two primary filters (Turner #110-811 and #110-822) and two secondary filters (Turner #110-824 and #110-823B) were employed. The range selector switch was adjusted to 30X and the excitation slit width to 7 mm. The emitted fluorescence was detected as peak areas on a Corning Model 841 chart recorder at a chart speed of 4 cm per min. Areas under the peaks were measured with a compensating polar planimeter. Quantification was achieved by comparing the areas of unknowns with those of the standard spotted on the same plate.

For the purpose of quantification, a standard curve was constructed with each run. Within phospholipid content limits of 1.5 to 4.0 μg, a linear curve was obtained. The variability in the standard curves obtained on four separate runs performed on different days and using different plates was calculated for each phospholipid amount from 1.5 to 4.0 μg. The co-
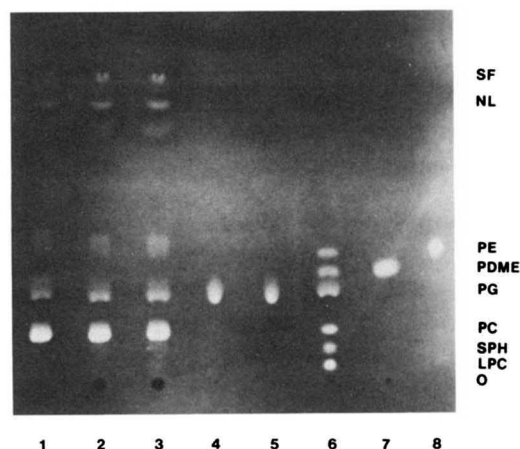

Fig. 1. Thin-layer chromatogram (Kieselgel plate) of extracted lipids from dog lung washings and reference phospholipid standards. Developing solvent chloroform–methanol–water 65:25:4 (v/v/v); the plate was sprayed with Rhodamine 6G 0.05% in 50% ethanol. Lanes 1–3: Total lipids from lung washings; 4–5: Reference phosphatidylglycerol (PG) standard (5 μg); 6: mixture of six phospholipid standards (Lyso-phosphatidylcholine (LPC) 15 μg, spingomyelin (SPH) 5 μg, phosphatidylcholine (PC) 5 μg, phosphatidylglycerol (PG) 5 μg, phosphatidyldimethylethanolamine (PDME) 5 μg, and phosphatidylethanolamine (PE) 5 μg); 7: PDME 15 μg; 8: PE 15 μg. SF = solvent front, NL = neutral lipids, O = origin.

efficient of variation (S.E./x̄ 100) in the fluorescence intensity for each amount of phospholipid was: 1.5 μg (3.6%); 2.0 μg (4.1%); 2.5 μg (2.8%); 3.0 μg (4.9%); 3.5 μg (5.4%); 4.0 μg (3.8%). The reproducibility expressed as mean S.E. coefficient of variation and tested on eight samples in triplicate, was 2.4 ± 0.7.

RESULTS AND DISCUSSION

The presence of PG as a major component of the surface-active material of the lung eluded early investigators. It had been many times erroneously identified as either phosphatidyldimethylethanolamine (PDME), phosphatidylethanolamine (PE), or "unknown phospholipid" due to difficulties in separating it from other phospholipids by TLC. PG is now widely accepted as the second major component of the lung surfactants (11–16). Its presence in the amniotic fluid in excess of 1% of the total PL is an important indicator of the fetal lung maturity. Gotelli et al (17) and Tsai and Marshall (18) have separated PG from the amniotic fluid by one-dimensional TLC and estimated the PG (percentage) by densitometry.

We have been able to separate PG in a solvent system reported here and to quantify it directly on TLC plates. The PG spots always chromatographed ahead of phosphatidylcholine (PC), but behind standard PDME and PE on Kieselgel plates (**Fig. 1**). A mixture
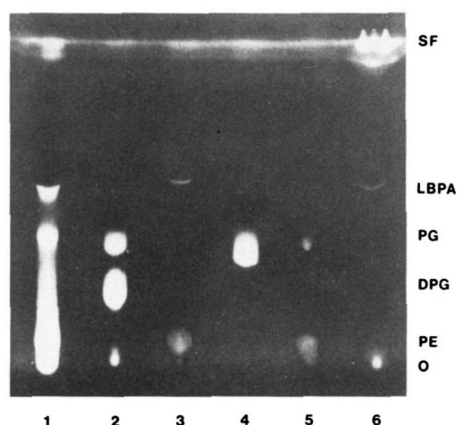
Fig. 2. Thin-layer chromatogram (Kieselgel plate) of phospholipids of rat liver, dog lung washings and reference phospholipid standards. Developing solvent was tetrahydrofuran–methylal–methanol–2 M ammonium hydroxide 10:8:2:1.1 (v/v/v/v); the plate was sprayed with Rhodamine 6G 0.05% in 50% ethanol. The phosphatidylethanolamine (PE) and the phosphatidylglycerol (PG) were separated initially by a one-dimensional system using chloroform–methanol–water 65:25:4 (v/v/v), eluted, and re-chromatographed using the second solvent system above. Lane 1: Rat liver PE; 2: Rat liver PG; 3: Dog lung washing PE; 4: Dog lung washing PG; 5: Standard PE and PG; 6: Rat liver lyso-bis-phosphatidic acid (LBPA). PE of both hepatic and pulmonary origin contained a second component with an R_f identical with that of LBPA.

of six PL standards (lysophosphatidylcholine (LPC), sphingomyelin (SPH), PC, PG, PDME, and PE) separated well on the Kieselgel plate, but in the lung washings the minor phospholipids, phosphatidylserine (PS) and phosphatidylinositol (PI) sometimes co-chromatographed with PC. Since our purpose was separation and quantification of PG, no effort was made to define the separation of all the phospholipids. Neutral lipids were always near the solvent front.

To evaluate the ability of the system to separate lyso-bis-phosphatidic acid (LBPA) we chromatographed the phospholipids of rat liver, a recognized source of LBPA (19). By use of our system, LBPA co-migrated with PE and not with PG, and was clearly separated from PE by the two-dimensional system utilizing tetrahydrofuran–methylal–methanol–2M ammonium hydroxide 10:8:2:1.1 (v/v/v/v) as the second solvent (13). Using extracts of dog lung washings, PE and PG were separated by our one-dimensional system, eluted, and re-chromatographed employing tetrahydrofuran–methylal–methanol–2M ammonium hydroxide 10:8:2:1.1 as the second solvent system. Again, PG migrated as a single component; a minor component with a R_f value identical with that of LBPA was clearly separated from PE (**Fig. 2**).

The purity of the separated PG spot was verified by two-dimensional TLC in five different solvent systems: (a) chloroform–methanol–concentrated ammonium hydroxide–water 130:60:5:4 (v/v/v/v); (b) chloroform–methanol–acetic acid–water 160:50:12:4; (c) tetrahydrofuran–methylal–methanol–water 10:6:4:1; (d) tetrahydrofuran–methylal–methanol–2M ammonium hydroxide 10:5:5:1; and (e) system (d) with the volume ratios of 10:8:2:1.1. Only one PG spot was detected in each of the solvent systems. The recovery of PG by one-dimensional TLC was 91%.

We preferred to use Rhodamine 6G, a very reliable fluorescent dye, for the fluorometry. Roch and Grossberg (20) quantitated five neutral lipid classes by direct fluorometry on homemade silica gel plates into which the Rhodamine 6G was incorporated. Nicolosi, Smith, and Santerre (21) also used Rhodamine 6G for their fluorometric estimation of four neutral lipids and PC. We have not noticed any quenching rings at the periphery of any PL spot using our method.

The PG content (mean ± S.E.) in washings derived from 11 lungs of 11 mongrel dogs was 236 ± 25 μg per g of parenchymal wet lung tissue and it accounted for $8.7\% \pm 2.3$ of the total PL. These results are in good agreement with other published data (11).

The large amount of PC compared to PG present in the lung washings may cause overloading problems. This problem can be avoided by reducing the volume of total lipids spotted so that the expected PG quantity is in the 1.5–4.0 microgram range. Consistent separation and R_f values of PG were observed with Kieselgel plates since they are affected less by high humidity and/or high room temperature. The absolute quantification depended on good PG standard and precise spotting.

The advantages of our method are: *1*) No lengthy preparatory steps and no separation of neutral lipids from phospholipids or of acidic from non-acidic phospholipids are necessary. The spotting is done directly from the extracted total lipids. *2*) The precoated Kieselgel plates are readily available from suppliers. *3*) One solvent system for a one-dimensional TLC development. *4*) Direct microgram quantification on the plates by fluorometry; tedious scraping and eluting of spots is avoided. *5*) Samples are not destroyed; they can be used for other determinations. *6*) Results can be obtained in 2 days after extractions of total lipids.

The role of PG in the lung surfactant system may be further clarified by the use of this method for its separation and quantification.

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