# Efficient elution of rabbit liver and plasma phospholipids from thin-layer plates

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SUMMARY The efficient recovery (96.3–99.6%) of phospholipids by elution from thin-layer plates is documented. The composition of phospholipids from rabbit liver and plasma is reported.

KEY WORDS phospholipids · quantification · thin-layer chromatography · rabbit · liver · plasma · sphingomyelin

In an earlier communication from this laboratory (1) a procedure was reported for determination of phospholipids by elution of thin-layer chromatography spots followed by P determination. The advantage of the procedure over similar methods of spot elution was the good quantitative recovery, due to the use of a special eluting mixture, of all individual phospholipids in amounts ranging from 0.1 to 15.0 μg of lipid P. De Bohner, Soto, and De Cohan (2) observed that the use of eluents composed of the commonly employed lipid solvents frequently resulted in poor general or specific phospholipid recovery. Abramson and Blecher (3) noted a selective loss of phosphatidyl serine in their system, and Skipski, Peterson, and Barclay (4) found only 1.8% sphingomyelin in rat liver which also suggested a selective loss of that compound.

The purpose of this communication is to provide more detailed proof of the dependability of the technique devised and to report a successful application of it to the analysis of phospholipids from rabbit liver and plasma. Interestingly, the reliability of the method was quite independently reported recently by Weinhold and Villee (5), who found that "recoveries of added lipid P of 95–98% were routinely obtained."

*Reference Materials.* Chromatographically pure standards were obtained as follows: lysophosphatidyl choline from General Biochemicals, Chicago, Ill.; sphingomyelin from Sylvana Chemical Company, Orange, N.J.; phosphatidyl choline from Sylvana (ex beef) and from Applied Science Laboratories, Inc., State College, Pa. (ex egg); phosphatidylinositol from Dr. H. Carter through the courtesy of Dr. E. Weber and from Sigma Chemical Co., St. Louis, Mo. (Folch Fraction I, beef brain); phosphatidyl serine from Applied Science Laboratories, Inc., and as a generous gift of Dr. Fauré, France (obtained through the courtesy of Dr. V. Skipski); dipalmitoyl cephalin from Mann Research Labs Inc., New York; L-α-dimyristoyl cephalin from La Motte Chemical Products Co., Chestertown, Md.; cardiolipin from Sylvana; phosphatidic acid from General Biochemicals; L-α-glycerophosphate from Sigma. Some compounds had to be further purified by preparatory TLC (1). Lysophosphatidyl choline and lysophosphatidyl ethanolamine were prepared from the fully esterified compounds by snake venom (6). All reagents used were of analytical grade (obtained from Fisher Scientific Company, New York) but were not redistilled.

*Methods.* Liver lipids were obtained by homogenization in 50 volumes of chloroform–methanol 2:1. Extracts were purified from nonlipid contaminants by the silicated paper method described previously (7). Blood was collected onto dry ethylenediamine tetraacetate disodium salt as the anticoagulant, 5–10 mg per ml of rabbit blood. Plasma lipid extracts were obtained and purified as previously described (8). Lipid extracts were concentrated to 3–10 μg of lipid P per μl in chloroform–methanol 1:1 and used as soon as possible with storage, when necessary, under nitrogen at −20°C.

*Procedure.* Basic plates 20 × 20 cm and 0.25 mm thick were prepared on the day of use according to Skipski, Peterson, Sanders, and Barclay (9). The silica had been washed twice with 5 volumes of chloroform–methanol 1:1. Several lanes 2 cm wide were drawn on the plate. As a rule three lanes were used for the quantitation and one for identification. The phospholipids were applied in volumes of 2–3 μl with a 5 μl Hamilton syringe of the fixed needle type fitted with a Chaney adapter (#7005 NCH). A micropipette, as used by Skipski et al. (4), necessitates the introduction of a correction factor for lipids left on the tip and is not recommended.

Ascending chromatography took place in a glass jar 12 × 4 × 11 inches. The developing solvent most commonly used consisted of chloroform–methanol–acetic acid–water 46:29:6:3; other solvents employed consisted of minor (1-volume) modifications of this one. Solvents described by Skipski et al. (9) were used in earlier experiments. Irrespective of the solvent used we encountered occasional difficulty in separating phosphatidyl serine from phosphatidyl inositol. This was also observed by others (5).

Spots were made visible by exposing the plate to iodine vapor. When most of the iodine had disappeared the spots were scraped off separately into stoppered 15 ml centrifuge tubes. Three blank areas were also removed. The lipids were eluted twice by shaking the tubes for 30 min at 40°C with 2 ml of the eluting mixture, ethanol–chloroform–water–acetic acid 100:30:20:2.

The whole procedure took 1 day. Appreciable loss of material resulted if separated lipids were left on the plate

This work was presented in part at the annual meeting of the Federation of American Societies for Experimental Biology, Chicago, Ill., April 1964 (1).

Abbreviation: TLC, thin-layer chromatography.

TABLE 1 RECOVERY OF PHOSPHOLIPID REFERENCE COMPOUNDS BY QUANTITATIVE TLC

| Compound | Amounts Applied | No. of Determinations | Mean ± SEM |
|---|---|---|---|
| | μg P | | |
| Lysophosphatidyl choline | 1.14 | 6 | |
| | 10.40 | 5 | 92.3 ± 1.5 |
| Sphingomyelin | 0.86 | 6 | |
| | 2.00 | 3 | 99.9 ± 3.3 |
| | 0.70 | 3 | |
| Phosphatidyl choline | 3.70 | 6 | |
| | 0.81 | 3 | 99.3 ± 2.4 |
| Phosphatidyl serine | 1.20 | 6 | |
| | 1.33 | 5 | 94.8 ± 3.3 |
| Dipalmitoyl phosphatidyl ethanolamine | 0.59 | 6 | |
| | 3.20 | 2 | 98.9 ± 4.9 |
| Dimyristoyl phosphatidyl ethanolamine | 1.25 | 6 | |
| | 1.32 | 3 | 93.6 ± 3.2 |
| Cardiolipin | 1.26 | 6 | |
| | 3.58 | 3 | 95.5 ± 3.4 |
| Mean | | | 96.3 ± 1.2 |

TABLE 2 COMPOSITION OF RABBIT PHOSPHOLIPIDS

| Compound | Plasma* (3.9–10.15)† | Liver* (3.65–12.25)† |
|---|---|---|
| Lysophosphatidyl choline | 2.5 ± 0.59‡ | 1.3 ± 0.33 |
| Sphingomyelin | 1.8 ± 0.40 | 4.5 ± 0.41 |
| Phosphatidyl choline | 80.2 ± 1.12 | 53.6 ± 1.80 |
| Lysophosphatidyl ethanolamine | 3.2 ± 0.18 | 0 |
| Phosphatidyl inositol | 3.9 ± 0.21 | 4.6 ± 1.52 |
| Phosphatidyl serine | 0 | 7.3 ± 1.94 |
| Phosphatidyl ethanolamine | 6.4 ± 0.80 | 25.4 ± 0.84 |
| Phosphatidic acid–cardiolipin | 2.1 ± 0.39 | 3.4 ± 0.65 |
| Recovery | 97.0 ± 2.58 | 99.6 ± 1.41 |

* Results from six animals. Three lanes on one plate for each animal.

‡ Per cent of lipid P ± SEM.

† Amounts applied (μg P).

overnight or if the spots were still stained with iodine before removal. Phosphorus was determined as described by Bartlett (10). Composition was calculated by averaging the percentages obtained from each lane.

*Results.* Recoveries of reference compounds were determined on amounts likely to be met with in biological samples. In some instances much larger and much smaller amounts were also used. Recoveries are shown in Table 1. Some loss of lysophosphatidyl choline occurred when very large amounts were applied. Since this could also be due to deacylation during storage, glycerol phosphate and inorganic sodium phosphate were applied to the plate in amounts equivalent to 2 μg of P. Following chromatography and extraction, P determinations showed recoveries of only 60.3% and 6.5%, respectively.

The composition of rabbit liver phospholipids (Table 2) demonstrated a preponderance of phosphatidyl choline and phosphatidyl ethanolamine. The general picture was not unlike that found in rat liver by this (1) and other authors (4, 11), except for small differences in the phosphatidyl serine and phosphatidyl inositol percentages. Rabbit plasma phospholipids, on the other hand, displayed several distinctive features. In comparison with human (1, 12) or rat (13) plasma, sphingomyelin was very low whereas phosphatidyl choline and phosphatidyl ethanolamine were rather high. These results are not in agreement with the report of Van Handel (14) who, using a combination of silicic acid column chromatography and of chemical determinations, reported values much closer to those in man.

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## REFERENCES

1. Biezenski, J. J. 1964. *Federation Proc.* **23:** 503.
2. De Bohner, L. S., E. F. Soto, and T. De Cohan. 1965. *J. Chromatog.* **17:** 513.
3. Abramson, D., and M. Blecher, 1964. *J. Lipid Res.* **5:** 628.
4. Skipski, V. P., R. F. Peterson, and M. Barclay. 1964. *Biochem. J.* **90:** 374.
5. Weinhold, P. A., and C. A. Villee. 1965. *Biochim. Biophys. Acta.* **106:** 540.
6. Rouser, G., J. O'Brien, and D. Heller. 1961. *J. Am. Oil Chemists' Soc.* **38:** 14.
7. Biezenski, J. J. 1962. *J. Lipid Res.* **3:** 120.
8. Biezenski, J. J. 1963. *Federation Proc.* **22:** 198.
9. Skipski, V. P., R. F. Peterson, J. Sanders, and M. Barclay. 1963. *J. Lipid Res.* **4:** 227.
10. Bartlett, G. R. 1959. *J. Biol. Chem.* **234:** 466.
11. Parker, F., and N. F. Peterson. 1965. *J. Lipid Res* **6:** 455.
12. Robinson, N., and B. M. Phillips. 1963. *Clin. Chim. Acta.* **8:** 385.
13. Newman, H. A. I., Ching-Tong Liu, and D. B. Zilversmit. 1961. *J. Lipid Res.* **2:** 403.
14. Van Handel, E. 1961. *J. Atherosclerosis Res.* **1:** 121.