MICROPROCEDURE FOR THE ISOLATION AND QUANTIFICATION OF PHOSPHOLIPIDS FROM TISSUE CULTURE DERIVED CELLS

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SUMMARY: A microprocedure allowing reproducible extraction and separation of nanomolar quantities of polar lipids from small numbers of cells grown in tissue culture is described. The method, using chloroform and methanol as extraction solvents ensures maximal sample recovery with minimal lipid manipulations, which allows additional identification of phospholipid subclasses on a submicroscale.

Key words: extraction; phospholipids; lipid subclass separation.

I. INTRODUCTION

Numerous lipid extraction procedures are based on the classical Folch et al. (1) or the Bligh and Dyer (2) techniques. These methods are adequate for routine use with lipid-rich cells or large quantities of tissues, however, they usually require both the need to pool multiple samples of lipid-poor material and the maintenance of correct solvent-to-water ratios. These are severe shortcomings when analyzing microgram amounts of individual sample lipid from slow growing tissue culture cells or plasma membrane isolates. Other procedures specifically designed for tissue culture cells (3,4) involve repeated transfer steps and unusual solvent mixtures, which can result in class-specific lipid losses. The procedure described here is a modification of the Folch et al. (1) and Suzuki (5) extraction methods, which can easily be applied to micromolar quantities of lipids. We illustrate the usefulness of this procedure by reporting the phospholipid composition of two human medulloblastoma cell lines in vitro.

II. MATERIALS

A. Equipment

- Tissue grinder, Duall, size 2, No. K885450, Kontes
- Centrifuge tubes, 50 ml disposable and 15 ml glass with Teflon lined screw caps
- Glass wool, pyrex
- Pasteur pipettes, 5¾ and 9 in length
- General purpose oven
- Refrigerated centrifuge capable of 500 x g
- Nitrogen, analytical grade with low pressure regulator
- Block heater or sand bath heater

B. Chemicals and solutions

- Silicic acid, Bio-Sil A, 100–200 mesh, No. 131-1340 Bio-Rad
- Methanol and chloroform, ACS grade
- Phosphate buffered saline (PBS), pH 7.2; Ca²⁺ and Mg²⁺-free
- 0.9% Saline

NOTE: Before use, all glassware should be acid washed by soaking overnight in 10 N HNO₃ or 10 NH₄SO₄

III. PROCEDURE

A. Cell transfer

1. Aspirate the medium from the culture dish and replace with 10 ml of ice-cold phosphate buffered saline (PBS) per 75 cm² area.
2. Using a rubber policeman, remove attached cells. Place the PBS-cell suspension in a 50 ml centrifuge tube and rinse the T-flask with three additional aliquots of cold PBS.
3. Centrifuge the cell suspension and combined rinses of Step 2 at 500 ×g × 15 min at 4°C.

4. Aspirate the supernatant fluid and wash the pellet twice with cold PBS. (This removes traces of serum.)

5. After Step 4, resuspend the cell pellet in 1 ml PBS and transfer using a Pasteur pipette to the tube portion of a preweighed ground glass tissue grinder. Rinse the centrifuge tube with 1 ml cold PBS and add to the tissue grinder tube.

6. Centrifuge the tissue grinder at 1000 ×g × 15 min at 4°C.

7. Aspirate the supernatant fluid and repeat Steps 5 and 6 twice to ensure quantitative transfer of harvested cells to the tissue grinder.

8. After the last wash of the centrifuge tube, weight the tissue grinder tube containing the cell pellet. Maintain the cell pellet at all times on crushed ice.

B. Lipid extraction

1. Add 20 vol of ice-cold CHCl₃:CH₃OH (2:1 vol/vol) to the volume of the cell pellet in the tissue grinder tube assuming 1 g = 1 cc. 

2. Homogenize the cell pellet for 2 min using hand rotation of the tissue grinder pestle. Vortex the tissue grinder for 1 min.

3. Repeat Step 2 continuously for at least 20 min.

4. Centrifuge the tissue grinder containing the cell homogenate for 10 × 1000 ×g at 4°C.

5. Carefully remove the supernatant fluid without disturbing the pellet using a Pasteur pipette. Place the supernatant fluid in a Teflon lined screw-capped 15 ml conical glass centrifuge tube.

6. Add 20 vol of ice-cold CHCl₃;CH₃OH (1:1 vol/vol) to the cell pellet in the homogenizer tube (based upon the initial cell pellet mass.)

7. Repeat Steps 2 through 6, combining the supernatants. Reextract the cell pellet a third time using 20 vol of ice-cold CHCl₃;CH₃OH (1:2 vol/vol). Repeat Steps 2 through 6.

8. Wash the extracted debris pellet in the homogenizer twice with 2 ml of cold CHCl₃;CH₃OH (2:1 vol/vol) using Steps 4 and 5.

9. Combine the supernatant fluids, noting the volume, and add an appropriate amount of cold CHCl₃;CH₃OH ratio (2:1 vol/vol). Vortex and note the final volume.

10. Add 0.9% NaCl (wt/vol) to bring the aqueous volume in Step 9 to 20% of the total solvent volume. Vortex.

11. Centrifuge 1000 ×g × 10 min to ensure phase separation.

12. Aspirate the upper phase and rewash the lower lipid phase as in Step 10 using Folch theoretical phase (CHCl₃;CH₃OH:H₂O (3:48:47 vol/vol). Do not remove the solvent-water interface in any of the washing steps.

13. The lipid residue may be stored until further use at −20°C or brought to dryness under a stream of N₂ and resuspended in a small amount CHCl₃;CH₃OH (2:1 vol/vol).

C. Lipid class separation

1. Place 0.3 g of activated (100°C, 12 h) silicic acid in a 15 ml conical glass centrifuge tube, add 5 ml methanol, and vortex.

2. Transfer the slurry to a 5/4 in Pasteur pipette preplugged with glass wool.

3. To the centrifuge tube in Step 1, add 5 ml methanol and transfer any additional silicic acid to the column. Packed columns may be stored in methanol until use.

4. Sequentially wash the silicic acid columns with 5 ml of CHCl₃, CH₃OH, and CHCl₃ and allow the last CHCl₃ wash to drain about 3 mm from the top of the column. Add the lipid to the sample from Part B, Step 13.

5. Elute the neutral lipid and cholesterol lipid fraction by adding 6 ml of CHCl₃ to the column, collecting the eluate into a 15 ml glass centrifuge tube.

6. Elute the phospholipids into another sample tube by adding 6 ml of methanol. Force out any remaining methanol by applying low pressure (2 psi) dry nitrogen.

7. The phospholipid column recovery can be determined by adding 15 μg phosphatidylcholine to the column after Step 6 and re-eluting the phospholipid with 6 ml methanol. Recovery should be in the range of 95 to 97%.

8. The collected fractions can be purged with N₂ and stored at −20°C or the solvent can be removed by evaporation under a stream of N₂ at room temperature or on a block heater maintained at 30°C.

9. Redissolve the phospholipid samples in a small amount of CHCl₃;CH₃OH (2:1 vol/vol) and store at −20°C under N₂.