Total Phospholipids and Phospholipid Subclasses Determination

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Abstract

Phospholipids are important lipid species in cell signal transduction. The assays of total phospholipids and phospholipid subclasses are important for signal transduction studies.

Reagents

• Sol I: 1.6 g of ammonium molybdate ((NH4)6Mo7O24□4H2O; M.W.=1235.86) is dissolved in 12 mL water.

• Sol II: 4 mL concentrated HCl and 1.0 mL of mercury are shaken with 8 mL of Sol I for 60 min and filtered through glass wool.

• Sol III: 20 mL of concentrated H2SO4 is added to the remainder of Sol I, then, Sol II is added to the resultant solution to give Sol III.

• Chromagenic solution: 45 mL methanol, 5 mL chloroform, and 20 mL water are added to 25 mL Sol III. It is stable for 3 months at 5 □C.

• CM mixture 2:1: chloroform: methanol w/BHT = 2 L: 1 L: 100 mg

• Mobile phase:

1. Use 1000 mL graduate cylinder to add 3 L of HPLC grade acetonitrile to a 4 L bottle;
2. Use 100 mL graduate cylinder to add 150 mL of HPLC grade methanol to the 4 L bottle;
3. Use 50 mL graduate cylinder to add 27 mL of HPLC (or ACS) grade phosphoric acid to the 4 L bottle;
4. Use 1000 µL automatic pipette to add 760 µL of to HPLC (or ACS) grade 98% sulfuric acid to the 4 L bottle;
5. Use stir bar to degas the mixture. Shake the bottle upside down vigorously several times.

• CM mixture:chloroform:methanol:butylated hydroxytolune (BHT) = 2L:1L:100mg.

• CM mixture:chloroform:BHT= 100 mL:900 mL:100mg.

Equipment

HPLC priming:

• Check if mobile phase is enough.

• Check if the washing solvent bottle in 507E autosampler is enough.

It is recommended to use degassed methanol as washing solvent (Ref 3: pp 2-9).

• Wash the autosampler syringe to get rid of air bubbles. Click the button of “507E wash” 2-3 times.

• Check if the autosampler tubing is leaking.

• Change to the method for use. Click “single run” botton. Check if the mobile phase line is in the right solvent bottle.

• Prime the pumps (First prime lines, then prime pumps).

• Check if batch file options are right. Check the shutdown method to make sure that it uses the same mobile phase as the running method.

• Check the hard disk space in Windows File Manager.

• Check if the waste solvent bottle is full.

Procedure

I. Total PL assay

1. Check the automatic pipette before sample pipetting. Add 100 µL lymph/bile/serum with automatic pipette into a 16x100 mm PTFE capped test tube, wash the pipette tip with 100 µL D.I. water, and add 4 mL of CM mixture with 5 mL dispenser.
2. Vortex the tube vigorously. Wait at least 1 h for proteins in the sample to denature. There will be white stuff emerging gradually.
3. Add 800 µL D.I. water into the tube. Shake the tube upside down 3 times. Wait until there are two clear layers. Siphon off the upper layer with the negative water pressure instrument.
4. Evaporate the tube under a gentle stream of N2 at 40 □C.
5. Add 400 µL chloroform to the tube. Cap tightly. Vortex the tube with Vortex-Genie gently 3 times at #3.
6. Add 100 µL chromagenic solution and shake the tube 3 times with fingers.
7. Cap the tube tightly. Otherwise the chloroform will evaporate and the absorbance will be very high. Boil the tube in the boiling water bath for 1 min 10 sec. Wait 5 min for the tube to cool down to room temperature.

8. Add 4 mL of chloroform gently into the tube and shake the tube 3 times with fingers. (Crucial point!!!)
9. Allow the tube to stand for 30 min at room temperature.
10. Take the lower solution quickly with a pasteur pipette.
11. Determine the absorbance at 710 nm. Use chloroform as the blank.

II. PL subclasses assay

1. Prepare a set of 16x100 mm tubes.
2. Use 100 µL autopipette to add 100 µL of lymph/bile/serum. Wash the pipette tip with 100 µL of fresh D.I. water.
3. Add 5 mL of CM (2:1) mixture to the tube. Vortex vigorously.
4. Use multiple automatic pipette to add 25 µL of concentrated HCl to lower PH. Lower PH to separate phospholipids from proteins and deactive proteins. Optimum amount of concentrated HCl needs to be determined in the future studies.
5. After overnight deproteinization, cotton-like residue will appear.
6. Add 1 mL of fresh D.I. water (20%). Shake gently upside down. Don’t vortex vigorously to break the protein residue to make sure easy separation of the residue.
7. Wait until two clear layers appear.
8. Siphon off the upper water layer.
9. Wait 30 min to let the leftover clear, then some white residue will appear on the surface, siphon off the residue again.
10. Evaporate the lower CM mixture layer. Check the temperature of the evaporator before using.
11. After drying, add 500 µL of inject solvent to dissolve the lipids. Methanol causes a negative peak close to PE peak. Acetonitrile causes a negative peak close to chloroform peak.

Too much chloroform is detrimental to the column and cause big solvent peaks. Small amount of chloroform is needed to dissolve the PI and PS.

To the best knowledge of this lab, acetonitrile (10):chloroform (1) (v/v) is considered the most suitable inject solvent.

12. Pour the final mixture into the autosampler vials for HPLC running.

The following document contains information on:

Setting up external standard curves (ESTD)
Method parameters
Composition of the mobile phase

HPLC methods

References

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Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

ESTD-method_parameters-mobile_phase.doc