Chapter 10

High-Throughput Assay of Secreted Phospholipases A_2 Inhibitors

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Abstract

Attempts to characterize, quantify, and/or modulate the activity of the secreted phospholipase A_2 family of enzymes result from the diversity of physiological roles for which these enzymes have been implicated. The 1-palmitoyl-2-(10-pyrenedecanoyl)-phosphatidylglycerol (pyrenePG)-based fluorometric assay is a sensitive and readily adaptable method for further elucidating phospholipase function under various experimental conditions, as well as a tool for screening chemical libraries for potent inhibitors of this enzymatic activity. This assay is based on the observed difference in fluorescent emission of pyrene aggregated in vesicles compared to sequestered in monomeric form by binding to bovine serum albumin after lipolytic activity, thus allowing direct quantification of hydrolyzed fatty acids by the measurement of the corresponding monomeric emission intensity. The assay can be carried out in multiwell plates for high-throughput screening of compound libraries.

Key words: Phospholipase A_2, Fluorometric assay, High-throughput screen, Interfacial enzymology, PLA_2, Fluorogenic, 1-palmitoyl-2-(10-pyrenedecanoyl)-phosphatidylglycerol, Secreted PLA_2 Inhibition Assay

1. Introduction

Phospholipases A_2 (PLA_2s) constitute an enzyme family that catalyzes the hydrolysis of the *sn*-2 ester bond of phospholipid substrates, producing free fatty acids and lysophospholipids (1). Physiological roles of PLA_2s have been shown to include the hydrolysis of esterified arachidonyl acyl chains from many distinct phospholipid membranes in a variety of tissues (2–4). This catalytic activity liberates free arachidonic acid, a precursor of prostaglandins, prostacyclins, leukotrienes, and other inflammatory-mediating eicosanoids created by downstream modification (2, 5–7).

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One early attempt to design a fluorescence-based phospholipase A_2 activity assay made use of a pyrenobutanoyl acyl chain at the *sn*-2 position of phosphotidylcholine (8). Later, phospholipid substrates with 10-pyrenedecanoyl acyl chains at the same position came into use (9). This chapter describes a similar assay with 1-palmitoyl-2-(10-pyrenedecanoyl)-phosphatidylglycerol (pyrenePG) as a substrate. A common aspect of these assays is that they are all based upon the varying emission maxima of pyrene excimers in vesicular form as opposed to monomeric pyrene sequestered by binding to excess bovine serum albumin (BSA) present in the aqueous phase. Only after the release of 10-pyrenedecanoic acid from the phosphatidylglycerol does the pyrene fluorophore desorb from the membrane vesicles to bind to BSA in the aqueous phase. By following the fluorescence of monomeric 10-pyrenedecanoic acid bound to albumin, the activity of phospholipases A_2 can be measured in real time.

2. Materials

2.1. Fluorescent Product and Substrate

2.1.1. 10-Pyrenedecanoic Acid (see Subheading 3.1)

1. C16:0 LysoPG (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phospho-(1′-*rac*-glycerol), Avanti, cat no. 858122P).
2. Acetone.
3. Round bottom flask.
4. 2,2-Dimethoxypropane.
5. Trifluoroacetic acid (CF_3COOH).
6. $CHCl_3$.
7. Methyl 10-chloro-10-oxodecanoate.
8. $AlCl_3$.
9. Pyrene.
10. Ice bath.
11. Separatory funnel.
12. HCl.
13. Vacuum distiller/Rotary evaporator.
14. Silica gel for flash chromatography.
15. Diethylene glycol (DEG).
16. Hydrazine hydrate (H_2NNH_2).
17. NaOH.
18. Nitrobenzene.
19. Reflux equipment.

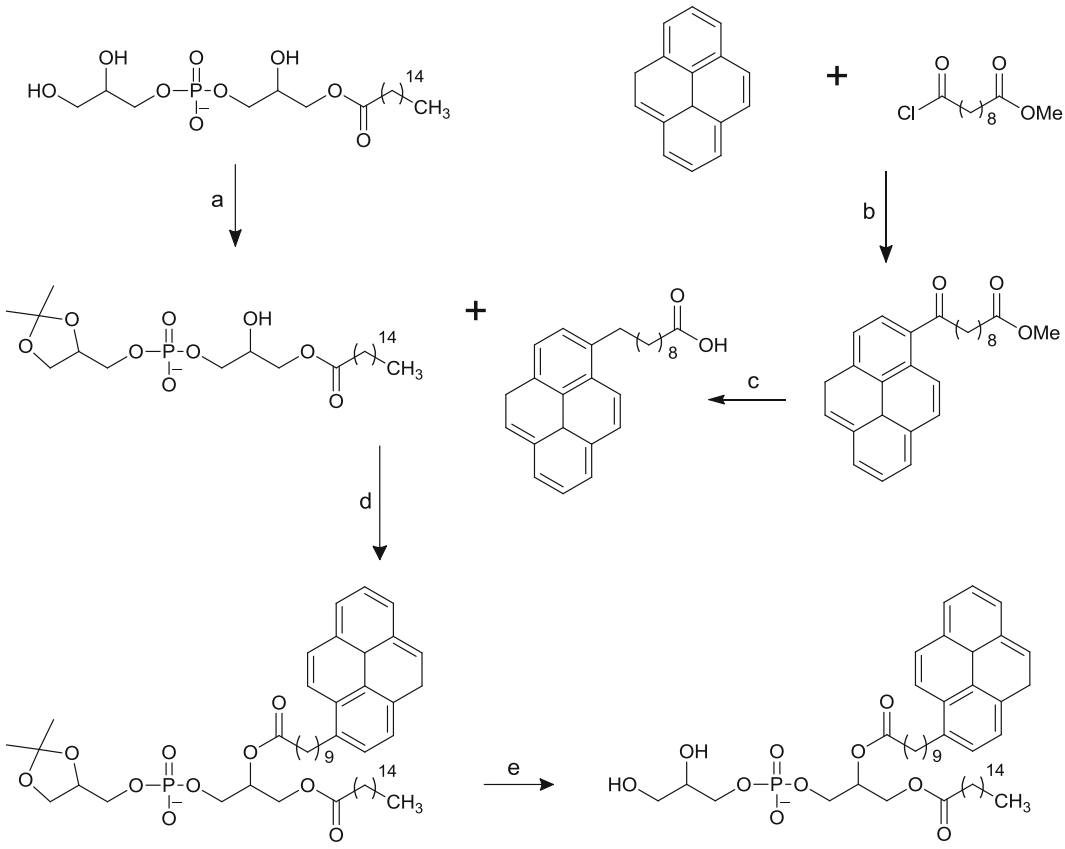

Fig. 1. Synthesis of 1-palmitoyl-2-(10-pyrenedecanoyl)-phosphatidylglycerol (pyrenePG). (**a**) 2,2-dimethoxypropane, CF_3COOH, acetone, rt, 60 h. (**b**) $AlCl_3$, nitrobenzene, rt, 18 h, 63% yield (**c**). H_2NNH_2, NaOH, DEG, 195°C, 6 h, 57% yield. (**d**) DMAP, Et_3N, $CHCl_3$, rt, 3 h, 48% yield. (**e**) $AcOH/H_2O$, $CHCl_3$, 60°C, 1 h, 74% yield.

2.1.2. PyrenePG (See Subheading 3.1 and Fig. 1)

1. 4-Dimethyaminopyridine (DMAP).
2. Triethylamine (NEt_3).
3. Dry chloroform ($CHCl_3$).
4. 2,4,6-Trichlorobenzoyl chloride.
5. H_2O.
6. MeOH/$CHCl_3$ solution: 20% methanol, 80% $CHCl_3$ (v/v).
7. Silica gel for flash chromatography.
8. AcOH solution: 80% acetic acid in H_2O.
9. Na_2SO_4.
10. Separatory funnel filter.
11. Round bottom flask.

2.2. Working Stock Solutions, Assay Buffer, and Experimental Solutions

This phospholipase A_2 assay takes place in a buffer imitating physiological conditions which may be made in large amounts and stored at 4°C for months. Three solutions—buffer with BSA, enzyme, or substrate—should be made from this original assay buffer daily before undertaking any experiments.

1. PyrenePG working solution: 100 μM pyrenePG (see Subheading 3.1) in 200 proof ethanol. Stored at −20°C and quantified before each use.
2. Assay buffer: 50 mM KCl, 1 mM $CaCl_2$, 50 mM Tris–HCl, pH 8.0. Filtered through a 0.22 μm sterile filter and stored at 4°C. Each well measured requires 300 μL of this buffer.
3. BSA solution: 3% fatty acid-free BSA (see Note 1) in assay buffer. Filtered through a 0.2 μm cellulose acetate sterile syringe filter, aliquoted, and stored at −20°C. Each 970 μL of assay buffer requires 30 μL of this solution before use.
4. Solution A: 30 μL BSA solution added to 970 μL assay buffer. Each well measured requires 100 μL of this solution.
5. Solution B: appropriate enzyme amount added to Solution A. Each well measured requires 100 μL of this solution.
6. Solution C: pyrenePG working solution added to assay buffer (no BSA) to yield 4.2 μM pyrenePG. Each well measured requires 100 μL of this solution.
7. Phospholipase A_2 enzyme(s).

3. Methods

Procedures and tasks related to this assay include: synthesis of product and substrate (see Subheading 3.1) and calibration of fluorimeter (see Subheading 3.2).

After the initial synthesis of pyrenePG substrate and 10-pyrenedecanoic acid product, these chemicals may be stored indefinitely at −20°C for future use. The 10-pyrenedecanoic acid product is necessary for calibrating the fluorimeter response.

3.1. Synthesis of Fluorescent 10-Pyrenedecanoic Acid Product and pyrenePG Substrate (See Fig. 1)

1. Suspend 185 mg, (0.36 mmol) of C16:0 LysoPG (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phospho-(1′-*rac*-glycerol), Avanti, cat no. 858122P) in 3 mL acetone in a round bottom flask.
2. Add 170 mL of 2,2-dimethoxypropane to the reaction mixture followed by addition of a catalytic amount of 99% CF_3COOH (100 μL).
3. Also add $CHCl_3$ until the lysoPG is fully dissolved.
4. Stir the reaction mixture until full conversion to acetonide protected product [as measured by ESI-MS in the negative mode

3.1.1. Acetonide Protection of LysoPG

(product peak MW: 523.3, starting material peak MW: 483.5)]. Full conversion to product requires about 60 h stirring at room temperature.

5. Stop the reaction by removing solvent by rotary evaporation to give a dark orange oil.
6. Dry the oil in vacuo for 30 min to remove most of the CF_3COOH.
7. Dissolve the product in dry 10–15 mL $CHCl_3$ (do not use wet $CHCl_3$ as this will react with the acetonide moiety), and remove the solvent by rotary evaporation. Repeat three times to remove CF_3COOH in the oily residue.
8. Dry the oily residue in vacuo overnight to give an oil (274 mg, 150% excess weight is likely due to unremoved TFA). ^{1}H NMR (300 MHz, , $CDCl_3$): 0.90 (t, J=6.3 Hz, 3H), 1.27 (s, 25H), 1.37 (s, 3H), 1.44 (s, 3H), 1.61 (m, 3H), 2.34 (t , J=7.2 Hz, 2H), 3.78 (m, 2H), 3.93–4.10 (m, 6H), 4.24–4.34 (m, 2H) MS (ESI neg. ion) *m/z*: 523.3 (M–1).

3.1.2. Methyl 10-oxo-10-(Pyren-3-yl)Decanoate

1. Dissolve methyl 10-chloro-10-oxodecanoate (265 µL, 2.0 mmol) in 4 mL nitrobenzene and stir at 0°C in an ice bath.
2. Add $AlCl_3$ (264 mg, 2.0 mmol) in portions over 1 min. After addition of all $AlCl_3$, stir the reaction mixture for an additional 2–3 min and then add pyrene (200 mg, 0.99 mmol) to the reaction mixture (reaction turns blood red color).
3. Remove the ice bath and stir the reaction mixture for 18 h at room temperature.
4. Pour onto 5 mL ice cold water in a separatory funnel. Acidify the water layer with 1 N HCl, and separate the layers.
5. Extract the water layer with 3×5 mL $CHCl_3$.
6. Combine the organic layers with the nitrobenzene layer.
7. Remove the $CHCl_3$ by rotary evaporation, and remove the nitrobenzene layer by vacuum distillation (6 mm Hg, 80°C).
8. Dissolve the yellow residue in 2–3 mL $CHCl_3$ and purify by flash chromatography over silica gel (100% $CHCl_3$) to give a yellow solid (250 mg, 63% yield). ^{1}H NMR (300 MHz, , $CDCl_3$): 1.33–1.46 (m, 8H), 1.61 (m, J=7.5 Hz, 2H), 1.86 (m, J=7.2 Hz, 2H), 2.29 (t, J=7.5 Hz, 2H), 3.21 (t, J=7.2 Hz, 2H), 3.65 (s, 3H), 8.03–8.33 (m, 8H), 8.85 (d, J=10.8, 1H).

3.1.3. Synthesis of 10-(Pyren-3-yl) Decanoic Acid

1. Dissolve methyl 10-oxo-10-(pyren-3-yl)decanoate (see Subheading 3.1.2) (2.9 g, 7.3 mmol) in 10 mL DEG followed by addition of 65% hydrazine hydrate (1.95 mL, 40 mmol) and NaOH (2.3 g, 50 mmol).

2. Reflux the reaction mixture for 2 h at 190°C.
3. Replace the reflux condenser with a distillation apparatus and stir the reaction at 195°C for 30 min to remove any low boiling solvents formed during the reaction.
4. Cool the reaction mixture to room temperature, and attach again the reflux condenser to the flask.
5. Reflux overnight at 190°C.
6. Cool to room temperature and add 8 mL H_2O.
7. Add concentrated HCl to pH XXX make the reaction mixture acidic.
8. Collect the yellow precipitate formed by vacuum filtration.
9. Dry the yellow precipitate in vacuo overnight and purify by flash chromatography (95% $CHCl_3$/5% MeOH) to give a light yellow solid (1.5 g, 57% yield). ^1H NMR (300 MHz, $CDCl_3$): 1.31 (s, 8H), 1.48 (m, 2H), 1.62 (m, 2H), 1.85 (q, J=7.8 Hz, 2H), 2.33 (t, J=7.5 Hz, 2H), 3.33 (t, J=7.8 Hz, 3H), 7.86 (d, J=7.8 Hz, 1H), 7.95–8.17 (m, 7H), 8.28 (d, J=9.3 Hz) MS (ESI neg. ion) *m/z*: 371.3 (M – 1).

3.1.4. Acetonide-Protected 1-Palmitoyl-2-(1-Pyrenedecanoyl)-sn-Glycero-3-Phosphatidylglycerol (Acetonide-Protected Pyrene PG)

1. Dissolve acetonide protected lysoPG (see Subheading 3.1.1) (244 mg, 0.45 mmol), 10-pyrenedecanoic acid (see Subheading 3.1.3) (415 mg, 1.1 mmol), 4-dimethyaminopyridine (330 mg, 2.7 mmol), and NEt_3 (220 μL, 1.6 mmol) in 15 mL dry $CHCl_3$ and stir at room temperature for 10 min.
2. Add 2,4,6-trichlorobenzoyl chloride (450 μL, 2.90 mmol) to the reaction mixture, and stir it overnight at room temperature.
3. After stirring overnight, add 500 μL H_2O to the reaction mixture to quench the acid chloride, and remove the solvent by rotary evaporation to give a crude oil.
4. Dissolve the crude oil in 2 mL MeOH/$CHCl_3$ solution and purified by column chromatography over silica gel (20% MeOH/80% $CHCl_3$).
5. Dry the product in vacuo to give a white/brown solid (190 mg, 48% yield). MS (ESI neg. ion) *m/z*: 878(M – 1).

3.1.5. 1-Palmitoyl-2-(10-Pyrenedecanoyl)-sn-Glycero-3-Phosphatidylglycerol (pyrenePG)

1. Dissolve acetonide-protected pyrenePG (see Subheading 3.1.4) (190 mg, 0.21 mmol) in 1 mL $CHCl_3$.
2. Add of 5 mL 80% acetic acid in H_2O.
3. Stir the reaction mixture for 1 h at 60°C.
4. Cool the reaction mixture to room temperature and remove the solvent by rotary evaporation.
5. Dissolve the crude amber solid in 2 mL MeOH/$CHCl_3$ solution and purify by column chromatography over silica gel (20% MeOH/80% $CHCl_3$).

6. Collect the fractions containing product and remove the solvent by rotary evaporation.
7. Redissolve the product in 4 mL $CHCl_3$ and pour into a separatory funnel containing 20 mL H_2O and 20 mL $CHCl_3$.
8. Pour the product into the separatory funnel over a cotton plug to remove any trace silica.
9. Wash the product with 2×20 mL H_2O.
10. Dry the layers separated over Na_2SO_4 and filter into a round bottom flask.
11. Remove the solvent to give an amber/white solid (74% yield). ^1H NMR (300 MHz, $CDCl_3$): 0.83 (m, 3H), 1.17–2.0 (m, 43H), 2.26 (m, 4H), 3.22 (m, 2H), 3.60–4.40 (m, 9H), 5.25 (m, 1H), 7.76–8.16 (m, 9H) MS (ESI neg. ion) *m/z*: 838.1 (M – 1).

3.1.6. PyrenePG Conservation

1. Dissolve dried pyrenePG in $CHCl_3$ at 1 mg/mL concentration.
2. Transfer 1 mL of the solution to an amber screw-cap vial and remove the solvent either in a centrifugal concentrator (Speed-vac) or by passing a stream of N_2 into the vial to give a dried powder.
3. Store the dried powder (≈1 mg/vial) in a storage jar at –80°C with desiccant. Typically, 1 mg of pyrenePG is enough to make about 90 mL of 4.2 μM pyrenePG for the assay.

3.2. Calibration of Fluorimeter

Following the procedures described in the rest of the methods section below, prepare and measure wells containing pyrenedecanoic acid in amounts similar to those expected to be obtained during enzymatic activity assays. Use the resulting fluorescent readout to create a standard curve for the fluorometric instrument. For our purposes, excitation and emission wavelengths of 342 and 405 nm, respectively, provided maximum sensitivity (see Note 2).

3.3. Preparation of pyrenePG Working Solution from dry pyrenePG Stock

1. Dissolve approximately 1 mg pyrenePG in 1 mL 1:1 toluene:ethanol in a round-bottomed glass culture tube.
2. Vortex and sonicate extensively to resuspend the reagent, using hand warmth to facilitate the process. Sonication could be performed with a bath sonicator (e.g., Laboratory Supplies Co., Inc., Hicksville, NY; 80 watt output; model G112SP1G). Avoid sonication of flat bottom vials containers as this may lead to shattering.
3. Remove the solvent by centrifugal evaporation (Speed-Vac), and resuspend the compound again in 2.5 mL pure ethanol, vortexing, sonicating, and heating with hands to aid the process as before.
4. Transfer the solution to two 1.5 mL microtubes, centrifuging to remove insoluble particles, and remove the supernatant for use in the assay.

3.4. Quantification of pyrenePG Working Solution

Determining the amount of active pyrenePG is necessary due to possible incomplete resuspension of the reagent in ethanol.

1. For calibration, use 1 mL of pure ethanol as a blank while measuring the absorbance at 342 nm.
2. Add 20 μL of pyrenePG working solution and measure again, applying the appropriate dilution factor to the absorbance measurement.
3. Repeat with differing amount of substrate to ensure a linear response if desired. The molar extinction coefficient of pyrenePG is approximately 40,000 cm^{-1}.

3.5. Optimization of Enzyme Amount Used per Well

The amount of enzyme used in each well should be enough to ensure a fluorescent change of at least ten times background. Therefore, care should be taken to first obtain a consistent value of background fluorescence change in the absence of enzyme: this number is usually significant and cannot be assumed zero. Next, the minimum amount of enzyme necessary to produce a monomer fluorescence emission of at least ten times background should be determined through trial and error (see Notes 3 and 4).

3.6. Addition of Solutions to 96-Well Plate

Since this assay ultimately measures fluorescence change over a short time period, care should be taken to ensure simultaneous reaction initiation in each well. This can be accomplished by using a multichannel micropipette or other high-throughput device to add substrate to wells already containing enzyme, an effective method since the large volume (100 μL; one-third of total well volume) added thoroughly mixes the well contents (see Notes 5 and 6).

For clarity, the order of addition of each experimental solution for control and inhibition wells is enumerated here:

Negative Control Well: 200 μL Solution A followed by 1 μL DMSO and 100 μL Solution C.

Inhibition Screen Well: 100 μL Solution A, 1 μL DMSO with inhibitor (see Note 4), 100 μL Solution B, 100 μL Solution C.

Positive Control Well: 100 μL Solution A, 1 μL DMSO, 100 μL Solution B, 100 μL Solution C.

3.7. Other Assays Using pyrenePG

Due to its ease of use, the pyrenePG assay may be used for a variety of purposes other than high-throughput screens (see Note 7).

4. Notes

1. Ensure that fatty-acid free BSA (e.g., Sigma A6003) is used.
2. Varying slightly the excitation and emission wavelengths may increase sensitivity on other instruments; the wavelengths

provided are optimal for our purposes. Measuring emission wavelengths significantly above 400 nm risks exposure to the fluorescent emission of pyrene excimers. The fluorimeter used in our laboratory is a Victor³ V Model 1420-040 by PerkinElmer, Inc. All assays were performed at room temperature (21°C) with ten 1.0 s measurements made per well. The excitation and emission filters used were P340 (10 nm bandwidth) and D405 (10 nm bandwidth), respectively.

3. Over the course of several assays, e.g., high-throughput screening, fluorescence change may decrease. This is usually due to actual attenuation of enzymatic activity; therefore, it behooves the scientist to use fresh enzyme dilutions as often as possible, keep these solutions on ice, store pyrenePG substrate in darkness, and/or intermittently measure fluorescence change values of positive control wells to use in data analysis. PyrenePG vesicles are stable on the benchtop for at least several hours.
4. Addition of even small amounts of organic solvents or detergents can drastically alter this assay through vesicle disruption. The pyrenePG stock concentration must be greater than 100 μM to prevent introduction of excessive amounts of ethanol. Organic solutions of inhibitors or compounds to be screened may lead to similar effects. Seemingly innocuous sources of contamination, such as detergents, present in enzyme stock solutions may have severely detrimental effects even at low concentration.
5. Alternatively, calcium may be added for the initiation of calcium-dependent enzymatic reactions. In this case, assay buffer lacking calcium should be made and used in the preparation of all solutions. Addition of a calcium chelator, such as EGTA, will ensure the absence of free calcium from the reaction mixture if desired, and enough calcium should be added in excess of the chelator at the initiation time point.
6. Since some fluorimeters measure the fluorescence signal from each well separately and serially, the overall elapsed time of different assays may vary depending on the total number of wells included. This often must be considered when making direct comparisons of experiments, and the best way to avoid any discrepancies is to measure the same number of wells during every experimental trial.
7. Examples include direct kinetic comparison of phospholipase activity on mixed vesicles, quantification of stock solutions of enzymes with predetermined velocities, determining the extent of overall enzymatic inhibition by small molecules or of active site-specific binding by potential active site inhibitors (10), and quantification of pyrenePG-containing vesicles for use in other assays. Additionally, one could conceivably synthesize pyrenePG substrates with the fluorescent moiety present at the *sn*-1 position for quantification of PLA1 activity.

References

1. Lambeau G, Gelb MH (2008) Biochemistry and physiology of mammalian secreted phospholipases A2. Annu Rev Biochem 77:495–520
2. Vogt W (1978) Role of phospholipase A2 in prostaglandin formation. Adv Prostaglandin Thromboxane Res 3:89–95
3. Irvine RF (1982) How is the level of free arachidonic acid controlled in mammalian cells? Biochem J 204:3–16
4. Blackwell GJ, Duncombe WG, Flower RJ et al (1977) The distribution and metabolism of arachidonic acid in rabbit platelets during aggregation and its modification by drugs. Br J Pharmacol 59:353–366
5. Bergstroem S, Danielsson H, Klenberg D et al (1964) The enzymatic conversion of essential fatty acids into prostaglandins. J Biol Chem 239:PC4006–PC4008
6. Murphy RC, Hammarström S, Samuelsson B (1979) Leukotriene C: a slow-reacting substance from murine mastocytoma cells. Proc Natl Acad Sci USA 76:4275–4279
7. Funk CD (2001) Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294:1871–1875
8. Hendrickson HS, Rauk PN (1981) Continuous fluorometric assay of phospholipase A_2 with pyrene-labeled lecithin as a substrate. Anal Biochem 116:553–558
9. Radvanyi F, Jordan J, Russo-Marie F et al (1989) A sensitive and continuous fluorometric assay for phospholipase A_2 using pyrene-labeled phospholipids in the presence of serum albumin. Anal Biochem 177: 103–109
10. Oslund RC, Gelb MH (2011) Biochemical Characterization of Selective Inhibitors of Human GIIA Secreted Phospholipase A2 and Hyaluronic Acid-Linked Inhibitor Conjugates (in preparation).