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Measurement of lysophosphatidic acid and sphingosine 1 phosphate by liquid chromatography-coupled electrospray ionization tandem mass spectrometry.

Maria P. Kraemer, Suchismita Halder, Susan S. Smyth, and Andrew J. Morris

Gill Heart and Vascular Institute, University of Kentucky College of Medicine, Lexington Veterans Affairs Medical Center, Lexington KY, USA, Tel: 859 323 3749, Fax: 859 257 3235

1. Phospholipids are sensitive to acid hydrolysis and there have been literature reports that LPA can be generated by acid hydrolysis of lysophospholipids during extraction methods using acidified organic solvents. In our experience, this is only seen with much higher concentrations of HCl in the extraction solvent system than the 0.1M concentration used in our method. To investigate this issue we have added a series of odd carbon chain phospholipids to plasma and attempted to detect the corresponding odd carbon chain LPA species using the LC MS methods described below with triple quadrupole or multistage high resolution instruments. In these studies levels of these LPA species were consistently below the detection limit of the instrument.

2. The assay procedure described above is optimized for analysis of biological fluids. It can be modified for extraction of lipids from small quantities (up to 100mg) of tissue by homogenizing the tissue in 0.5 ml of 0.1M HCl and then following the procedure from 2 omitting steps 4 and 5. We use a Tekmar tissumizer homogenizer for this purpose with a disposable micro tip.

3. In general measurements of these lipids in biological fluids are normalized to the starting volume of the sample. For measurements in tissues lipid levels can be normalized to lipid phosphorous measured after wet digestion in perchloric acid or by measuring levels of more abundant lipids, for example phosphatidylcholines[27,28].

4. Because these the mass spectrometry method for S1P measurement is monitoring no more than 8 separate MRM transitions a 150 ms dwell time results in at least 9 data points across each peak which provides accurate and sensitive quantitation. On the 4000 Q-Trap instrument the limit of quantitation of this assay is ~10 fmol of S1P on the column. The limit of detection is ~1 fmol of S1P. The assay is linear up to ~10 pmol of S1P on the column. These assay parameters would have to be determined empirically on the particular instrument system.

5. Avanti Polar Lipids sell a deuterated S1P standard that can be substituted for C17 S1P in the above method and used for quantitation of S1P using standard stable isotope dilution methods.

6. We have validated these methods for use with human and mouse plasma and some tissues and other biological fluids. As with all LC MS methods validation using different matrices would be needed before the methods could be used with confidence to analyze other samples.

7. Our standard LPA method on the 4000 Q-Trap uses 100 ms dwell times which results in at least 7 data points across a chromatographic peak even when measuring 15 different LPA species. The limit of quantitation for C17 LPA is ~10 fmol LPA on the column with a limit of detection of ~1fmol. The method is linear up to 10 pmol LPA on the column.

8. The sensitivity of this method for different LPA species is highly dependent on the chain length and degree of saturation, presumably because of the differential efficiency with which these species are ionized, are stable during ionization and transmission through the instrument. Specifically, the instrument response to short chain saturated LPA species can be 2–3 fold higher than to longer chain saturated species. Generation of LPA species specific calibration curves is therefore important if absolute quantitation of LPA species is desired.

9. These methods have been validated for measurement of LPA in mouse and human plasma and would need to be independently validated if used with other samples and matrices.

10. It has been suggested that LPA can be formed by in source fragmentation of some anionic lysophospholipids, predominantly lysophosphatidylserine and lysophosphatidylinositol and that this phenomenon would lead to overestimation of the true levels of LPA in the sample. Using the instrument settings described in this method with a modified chromatographic method that separates different classes of lysophospholipids we found that ~1% of lysophosphatidylinositol and up to 5% of lysophosphatidylserine are fragmented to generate LPA consequently in our hands because plasma levels of these other lysophospholipids are lower than levels of LPA we do not consider this to be a significant issue. Separation of these different lysophospholipid classes can be achieved by modifying the chromatographic method to employ a 10 minute linear gradient from solvent A to solvent B. This would be important if analyzing samples that contain higher levels of other anionic lysophospholipids.

11. It is well established that lysophosphatidic acid can be formed in biological fluids ex vivo through the actions of autotaxin on lysophospholipids which are abundant in these systems[11]. Although not directly relevant to the analytical methods discussed here rapid extraction of plasma or storage on ice before extraction is important to minimize this process.

Abstract

Lysophosphatidic acids and sphingosine 1 phosphate are bioactive lipids that regulate diverse cellular and physiological processes through actions that are largely mediated by cell surface receptors. The roles played by these lipids in multiple disease processes makes the enzymes and receptors involved in their synthesis, inactivation and signaling attractive targets for pharmacological therapies. In this chapter we describe methods for sensitive accurate quantitation of LPA and S1P levels in biological fluids using liquid chromatography coupled electrospray ionization tandem mass spectrometry.

Keywords

lysophospholipids; mass spectrometry; liquid chromatography

1. Introduction.

LPA and S1P are bioactive lipid mediators that exert largely or wholly cell surface receptor mediated effects on a wide variety of cells. Studies using genetic and pharmacological approaches in animal models implicate LPA and S1P in a broad range of physiological and pathophysiological processes including inflammation, cancer, cardiovascular disease, pain, and neurological injury [1–8]. While both of these lipids can be generated intracellularly as intermediates in pathways of lipid synthesis and degradation they are also both present in extracellular biological fluids including cerebrospinal fluid, lymph and blood plasma[9]. Although other mechanisms are possible the most parsimonious theory holds that these extracellular pools of LPA and S1P are the source of biologically active “signaling” lipids. LPA can be generated extracellularly by the secreted lysophospholipase D autotaxin and possibly also by pathways that involve A-type phospholipases[10,11]. S1P has to be formed intracellularly by ATP dependent kinases and mechanisms for secretion of intracellular S1P involving various plasma membrane transporters have been reported[7]. Because LPA and S1P are not water soluble they are necessarily bound to “chaperone” molecules in biological fluids. This has been best studied in plasma where both lipids show associations with serum albumin and with circulating lipoproteins. In the case of S1P a specific association with apolipoprotein M mediates association with high density lipoprotein. There is evidence that HDL/apoM associated S1P has distinct signaling functions from albumin bound S1P and indeed the concept that binding of these lipids to plasma proteins and protein lipid complexes protects them from degradation and may influence their signaling properties is particularly interesting[12]. Because LPA and S1P are involved in multiple pathological processes including tumor growth and metastasis, fibrosis, vascular inflammation and atherosclerosis, neuropathic pain and brain injury and immune system function the enzymes and receptors involved in synthesis and signaling by these bioactive lipids are attractive targets for development of small molecule and perhaps also antibody therapeutics [13]. Studies of LPA and S1P metabolism and signaling in preclinical and clinical models are critically dependent on the availability of analytical methods to enable their precise detection and quantitation at low (sub pmol) levels in complex matrices that often contain other lipids at far higher (micro to milli molar) levels. Mass spectrometry methods primarily using

reverse phase HPLC coupled with electrospray ionization and selected ion monitoring approaches using triple quadrupole instruments have been widely used to measure LPA and S1P[14–24]. Literature methods for measurement of these lipids are largely very similar and primarily differ in the choice of column and associated solvent system. We have used these approaches to measure the activity of LPA and S1P generating and degrading enzymes, to study LPA and S1P metabolism in isolated cells and to examine the impact of genetic and pharmacological approaches targeting LPA and S1P metabolism and signaling in preclinical models and clinical settings. Although the basic approach used in our laboratory for these assays has been consistent we have continued to improve these methods to address a number of issues that have arisen in the literature that have been suggested to confound these measurements. This includes suggestions that LPA can be formed by hydrolysis of phospholipids during acidic extractions[25], that LPA can be formed by ion source fragmentation of anionic lysophospholipids during electrospray ionization[26]. The most persistent limitation of these measurements remains sample to sample carry over which can be significant for these lipids and has a major impact on assay precision and robustness. In this brief chapter we discuss our approaches to these measurements and present standard approaches currently used in our laboratory.

2. Materials.

2.1 Mass spectrometry instrumentation.

We have used ABSciex hybrid triple quadrupole linear ion trap instruments coupled with Shimadzu chromatography equipment for these measurements. Specifically the methods described below have been developed and validated using ABSciex 4000 Q-Trap instruments with an ABSciex Turbo V ion source and a Shimadzu Prominence binary HPLC system with an autosampler and column oven and an ABSciex 6500 plus Q-Trap instrument with an ion drive ion Turbo V ion source and a Shimadzu Nexera X2 UPLC system with a rack changing auto sampler and column oven. The 4000 series instruments are configured with a switching valve between the column and ion source that enables diversion of the column flow to waste. The 6500 series instrument has an integral switching valve. Use of the switching valve during sample loading and equilibration reduces contamination of the ion source improving assay sensitivity and robustness. Instruments of this type are expensive to acquire, maintain and operate so it is likely that investigators seeking to use these methods may not have access to identical instrumentation and may have to work with shared or facility core owned and operated instrumentation. All of the methods described below could be set up on HPLC coupled triple quadrupole mass spectrometer systems with electrospray ionization capabilities. As explained below the accuracy and sensitivity of these measurements depend on the speed and sensitivity of the instrumentation, particularly the rate at which data can be collected in selected ion monitoring mode which is the primary determinant of the accuracy of peak integration. Accordingly assay performance (i.e. limits of detection, quantitation and accuracy) on other analytical platforms would have to be evaluated on an instrument specific basis.

2.2 Solvents, tubes, internal standards.

Extractions are performed in 8ml borosilicate glass tubes with Teflon lined caps which are available from multiple commercial sources. Solvents and other reagents are HPLC grade and again available from many suppliers. Internal standards can be purchased from Avanti Polar Lipids, Alabaster AL.

2.3 Other equipment.

Thorough mixing of the samples before and after phase separation is important. We use a Tallboys Multi Tube Vortexer, Troemner Inc, Thorofare NJ, USA for efficient mixing of batches of tubes. Any low speed centrifuge with a swinging bucket rotor and adapters for the extraction tubes is suitable for centrifugation of the extraction tubes. For evaporation of solvents we use a Zymark/Biotage Turbo Vap concentrator (Biotage AB Charlotte, NC USA) that has been adapted for use with 4ml vials although an N-evap (Organomation Inc, Berlin MA) with Pasteur pipettes is an effective substitute. We use a Parker/Balston NitroVap membrane nitrogen generator connected to an air compressor to generate nitrogen gas for these evaporators but these methods would also work with tanked/compressed nitrogen gas. Dispensing of samples, internal standards and resuspension of extracted material for analysis requires calibrated air displacement pipettes.

3. Methods.

3.1 Lipid Extraction from biological fluids-blood, plasma, cell culture media

1. Label 8 ml borosilicate glass tubes with Teflon lined cap
2. Add 2mls MeOH, 1 ml CHCl_3 to each tube
3. Add internal standard to tube (generally 50 pmol C17 LPA or C17 S1P i.e. 50 μl of a 1 μM solution in MeOH)
4. Add the sample to the tube. (for blood or plasma we extract 50 μl , for other biological fluids the volume can be larger up to the total aqueous volume limit of 0.5ml).
5. Add 0.1 M HCl so the total aqueous volume is 0.5 ml (i.e. for 50 μl blood add 0.45 ml 0.1 M HCl)
6. Vortex for 5 minutes. This will generate a single phase.
7. Add 1 ml CHCl_3 and 1.3 ml 0.1M HCl to separate phases
8. Vortex for 5 minutes
9. Centrifuge at $>3,000 \times g$ for 10 minutes. This will generate two phases with a "cake" of denatured protein and other insoluble materials at the interface.
10. Transfer lower phase to 4ml screw cap vial using a Pasteur pipette changing the pipette between samples. Take care to avoid the protein interface and upper phase. Leave behind ~50 microliters (don't worry about being more accurate than this because there is an internal standard to correct for recovery). It is important not to transfer the protein cake material at the interface into the vial

because this impedes effective reconstitution of the sample and can result in insoluble material being introduced into the chromatography system.

11. Evaporate to dryness under N₂
12. Resuspend/dissolve dried material in 0.1 ml MeOH, vortex and allow to stand for ~10 min
13. Transfer to auto sampler vial for LC MS analysis. We use 8mm 100 µl polypropylene vials with Teflon septa. The choice of vial needs to be consistent with the LC system auto sampler.

3.2 Measurement of S1P

HPLC method: Column: Agilent Zorbax Eclipse XDB C8 column, 5 µm, 4.6 × 150 mm

Solvent A: 75/25 of methanol/ water with formic acid (0.5%) and 5 mM ammonium formate (0.1%).

Solvent B: 99/1 of methanol/ water with formic acid (0.5%) and 5 mM ammonium formate (0.1%).

Flow rate: 0.5 mL/min; Injection volume: 10 µL; Oven temperature: 30 °C

MS parameters: Positive mode electrospray ionization, AB Sciex 4000 Q-Trap
Instrument settings—Declustering potential (DP): 61.0 V

Collision Energy (CE): 23.0 V

Collision Cell Exit Potential (CXP): 16.0 V

Ion spray voltage: 5500 V; Curtain gas: 20 Psi; CAD: Medium

Temperature: 550.0 °C; Gas 1: 40.0 psi; Gas 2: 40.0 psi

1. Analysis of S1P is conducted using a Shimadzu UFLC coupled with an ABI 4000-Qtrap hybrid linear ion trap triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode.
2. C17 S1P is used as an internal standard.
3. Samples are analyzed using an Agilent Zorbax Eclipse XDB C8 column, 5 µm, 4.6 × 150 mm column. The mobile phase consists of 75/25 of methanol/ water with formic acid (0.5%) and 5 mM ammonium formate (0.1%) as solvent A and 99/1 of methanol/ water with formic acid (0.5%) and 5 mM ammonium formate (0.1%) as solvent B. For the analysis of S1P and DH-S1P the separation is achieved using a gradient of 0% B for 1 min, 0% B to 100% B in the next minute, maintained at 100% B for the next 10 min and equilibrated to the starting conditions in 3 min (Table 1)
4. The flow rate is 0.5 mL/min and the column temperature is 30 °C.

5. The sample injection volume is 10 μ L.
6. The mass spectrometer is operated in the positive electrospray ionization mode with optimal ion source settings determined by synthetic standards of S1P, DH-S1P and C17 S1P with a declustering potential of 61 V, entrance potential of 10 V, collision energy of 23 V, collision cell exit potential of 16 V, curtain gas of 20 psi, ion spray voltage of 5500 V, ion source gas1/gas2 of 40 psi and temperature of 550 °C. MRM transitions are shown in table 3.
7. Quantitation of the indicated S1P species is achieved by normalization to the internal standard and reference to external calibration curves generated using authentic standards.

3.3 Measurement of LPA

HPLC method.—Column: Machery-Nagel Nucleodur C8 Gravity column; particle size: 5 μ m; length: 125 mm; internal diameter: 2.0 mm.

Solvent A: 75/25 of methanol/ water with 0.5 % formic acid and 0.1 % 5 mM ammonium formate.

Solvent B: 80% (99/1 of methanol/ water with 0.5 % formic acid and 0.1 % 5 mM ammonium formate) + 20% (Chloroform).

Flow rate: 0.5 mL/min; Injection volume: 10 μ L

Oven temperature: 30 °C

Instrument settings—Declustering potential (DP): –90.0 V

Collision Energy (CE): –26.0 V

Collision Cell Exit Potential (CXP): –11.0 V

Curtain gas: 20 psi

Collision Gas: Medium

Ion spray voltage: –4500 V

Temperature: 550.0 °C

Gas 1: 40.0 psi; Gas 2: 40.0 psi

1. Analysis of LPA is conducted using a Shimadzu UFLC coupled with an ABI 4000-Qtrap hybrid linear ion trap triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode.
2. C17 LPA is used as an internal standard.
3. Samples are analyzed using a Machery-Nagel Nucleodur C8 Gravity column; particle size: 5 μ m; length: 125 mm; internal diameter: 2.0 mm. The mobile phase

consists of 75/25 of methanol/ water with formic acid (0.5%) and 5 mM ammonium formate (0.1%) as solvent A and 80% (99/1 of methanol/ water with 0.5 % formic acid and 0.1 % 5 mM ammonium formate) + 20% (Chloroform). as solvent B.

4. The separation is achieved using a gradient of 0% B for 1 min, 0% B to 100% B in the next minute, maintained at 100% B for the next 6 min and equilibrated to the starting conditions in 3 min (Table 2)
5. The flow rate is 0.5 mL/min and the column temperature is 30 °C.
6. The sample injection volume is 10 µL.
7. The mass spectrometer is operated in the negative electrospray ionization mode with optimal ion source settings determined by tuning with synthetic standards of LPA species with a declustering potential of -90V, entrance potential of -10 V, collision energy of -26 V, collision cell exit potential of -11 V, curtain gas of 20 psi, ion spray voltage of -4500 V, ion source gas1/gas2 of 40 psi and temperature of 550 °C. LPA species specific instrument settings can be refined by tuning with authentic synthetic standards. LPA species specific MRM transitions are shown in table 4:
8. Quantitation of LPA species is achieved by using the internal standard and reference to calibration curves generated using authentic standards.

3. Concluding comments.

Standard operating procedures (SOPs) are critical to quality management in any analytical laboratory. The information described in this chapter can be used to develop SOPs that are specific to the laboratory adopting the methods. The SOP needs to separate process based quality assurance components, for example validation of instrument/equipment performance (i.e. Instrument tuning and calibration) from assay specific quality control (e.g. use of internal standards). The major complication with these methods is carry over between samples. This can be minimized by washing the auto sampler needle and port and monitored by inclusion of solvent blanks in the sample batches. We have observed batch to batch variation in carry over between HPLC columns which may relate to differences in end capping of the silica column matrix. In ongoing work we have found that this carry over issue is further mitigated by use of columns with a polymeric silica matrix, for example Waters bridged ethylene silica columns. We hope to publish revised methods using this type of column and a UPLC system that provide greater sensitivity than the methods reported here in the coming year. Finally, the sample preparation method described here is robust and efficient but cannot be simply adapted for automated sample processing because of the phase separation step and the need to recover the lower phase. In our hands, supported liquid extraction methods that absorb the aqueous and insoluble materials using diatom based supports do not result in efficient recovery of lipids although as newer reagents of this type become available this approach is certainly worth considering. Alternate sample preparation methods using methanol or butanol based extractions in which the lipids partition into the upper phase of the system offer a strategy to automate the sample preparation using standard

liquid handling approaches with adaptation of these methods to use microplate formats an obvious strategy to increase sample throughput[27,28].

5. References

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Table 1.

Gradient elution for SIP measurements

Time (min)	%B
0	0
1	0
2	100
11	100
12	0

Table 2.

Gradient elution for LPA measurements

Time (min)	%B
0	0
1	0
2	100
6.0	100
7.0	0

Table 3

Precursor product ion pairs for SIP measurements

	Q1 m/z	Q3 m/z
C17-S1P	366.1	250.2
	366.1	82.0
	366.1	79.0
DH-S1P	382.1	284.2
	382.1	266.1
	382.1	95.0
S1P	380.1	264.1
	380.1	82.1

Table 4.

Precursor product ion pairs for LPA measurements

	Q1 m/z	Q3 m/z
LPA 12:0	353.2	153.0
LPA 14:0	381.2	153.0
LPA 16:0	409.2	153.0
LPA 16:1	407.2	153.0
LPA 17:0	423.3	153.0
LPA 18:0	437.3	153.0
LPA 18:1	435.3	153.0
LPA 18:2	433.2	153.0
LPA 18:3	431.2	153.0
LPA 19:0	451.3	153.0
LPA 20:0	465.3	153.0
LPA 20:2	461.3	153.0
LPA 20:3	459.3	153.0
LPA 20:4	457.2	153.0
LPA 20:5	455.2	153.0
LPA 22:0	493.3	153.0
LPA 22:2	489.3	153.0
LPA 22:4	485.3	153.0
LPA 22:5	483.3	153.0
LPA 22:6	481.2	153.0