Lipid Mediator Metabolomics via LC-MS/MS Profiling and Analysis

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Abstract

Solid phase extraction coupled with liquid chromatography tandem mass spectrometry provides a robust and sensitive approach for the identification and quantitation of specialized pro-resolving mediators (lipoxins, resolvins, protectins and maresins), their pathway markers and the classic eicosanoids. Here, we provide a detailed description of the methodologies employed for the extraction of these mediators from biological systems, setup of the instrumentation, sample processing and then the procedures followed for their identification and quantitation.

Running header: Lipid Mediator Profiling

Key Words: Lipid mediator metabololipidomics, flux analysis, eicosanoids, resolvin, protectin, maresin, profiling, liquid chromatography-tandem mass spectrometry.

1. Introduction

The role of lipid mediators in regulating distinct aspects of the body's functions in humans and experimental systems is well appreciated [1-4]. In this chapter, we shall detail the methodologies pioneered in the Serhan laboratory to obtain a snapshot of the dynamic pathways for the four major bioactive metabolomes that include the arachidonic acid eicosapentaenoic acid and docosahexaenoic acid metabolomes [5-12]. These methodologies provide insights into mechanisms activated during inflammation as well as new leads into underlying causes of disease by measuring the flux down each of the major bioactive metabolomes [5-11]. The methodologies that will be discussed herein employ C18 based extraction to enrich for lipid mediators in biological systems. These are then coupled with reverse phase liquid chromatography electrospray tandem mass spectrometry that allows for the separation, identification and quantitation of these molecules [6,10]. Given that the structures of these mediators are conserved throughout evolution, these methodologies are applicable to biological material from experimental systems such as tunicates [13], mice [5,7] and baboons [9] as well as humans [6,8,14,15,11]. Thus, they facilitate the direct translation of findings made in experimental systems to humans and *vice versa*.

2. Materials

All solvents should be LC/MS or analytical grade. All stocks should be handled using zero dead volume Hamilton syringes. Ensure that the Hamilton syringes are appropriately cleaned using isopropanol and methanol; this should be done by aliquoting at least 20 syringe volumes with each solvent. Please note that these compounds are sensitive to light, oxygen and heat. They should be stored at -20 °C or -80 °C, and exposure to light should be minimized. Each time the stock is used, purge the vial with a gentle stream of nitrogen very briefly before closing it to

prevent oxidative degradation. DO NOT use these compounds with DMSO because the SPM are sensitive to isomerization and oxidation in this solvent.

2.1 Sample preparation and Solid Phase Extraction

1. Methanol (MeOH) is used for protein precipitation and sample extraction as well as solid phase extraction (SPE) cartridge equilibration

2. For washing of SPE cartridges use *n-hexane*

3. Methyl formate is used as SPE elution solvent

4. Isolute 500 mg / 3mL C18 SPE columns are used(Biotage)

5 Deuterium-labelled internal standards used for quantification can be found inTable 1.

1.

2.

2.2 Liquid chromatography tandem mass spectrometry

1. Water containing 0.01 % acetic acid is used as solvent A

2. MeOH containing 0.01 % acetic acid is used as solvent B

3. LC-MS standards (see Table 2-4)

4. A Poroshell 120 EC-18 4.6 mm ×100 mm × 2.7 µm reversed phase column is employed (Agilent)

3. Methods

3.1 Preparing standard mixes for internal standards and standard curves

3.1.2 Determining the concentration of synthetic material

1. Blank the UV spectrophotometer using the solvent of interest.
2. Aliquot a known volume of the stock using a Hamilton syringe
3. Measure the UV absorbance ensuring that a characteristic chromophore is observed for the molecule of interest (i.e. diene, triene or tetraene) [6]
4. Calculate absorption using a 3 point dropline
5. Calculate concentration using Beer-Lambert law:

Concentration = absorbance/extinction coefficient*dilution (*See Table 5 for λ_{max}^{MEOH} and extinction co-efficients for each conjugated double bond system*)

3.2 Tuning of the LC-MS-MS for Lipid mediator profiling

1. Using synthetic standards, first tune the probe position to maximize signal
2. Next, adjust electrode position
3. Afterwards, in the instrument parameters window adjust curtain gas, collisionally activated dissociation (CAD) gas, electrode voltage, source temperature and the source gases one at a time to identify optimal source settings.
4. Subsequently tune the compound parameters (Declustering Potential, Entrance Potential, Collision Energy, and Collision Cell Exit Potential) for each compound and for each transition
5. It is suggested that for each compound (both pathway markers & mediators) at least 2 transitions are used

3.3 Automated Lipid Mediator extraction

3.3.1 Sample preparation

1. Ensure that samples have not been freeze-thawed, fresh samples are preferred
2. For frozen samples allow to defrost on ice
3. For tissues dissociate gently in ice-cold MeOH ensuring that the samples remain cold throughout the process.
4. For liquid samples (e.g. cell culture preparations and plasma) add 4 equal volumes of ice-cold methanol containing deuterium-labelled internal standards to each sample
5. Place at -20 °C for 45 min to allow for protein precipitation
6. Centrifuge samples at 2000 ×g for 10 min at 4 °C
7. Collect supernatant
8. Transfer tubes to TurboVap Evaporator
9. Ensure that the water bath is set to 37 °C
10. Turn nitrogen feed on and set the flow rate to no more than 15 psi
11. Ensure that the lid is closed
12. Methanol volume should be evaporated to less than 1 mL using a steady nitrogen stream
13. Centrifuge samples at 2000 × g for 10 min at 4^{o}C
14. Samples are now ready for extraction. At this stage samples should not be stored

3.3.2 Lipid Mediator Extraction

1. Add new C18 columns on the rack
2. Add the collection plates containing elution tubes
3. Make sure run through plate is at position D
4. Click “run single method”

5. Select the extraction method
6. Click “prepare run”
7. Go to solvent feeder (5) and prime each individually (make sure, the containers are filled, especially the MF)
8. Go to extraction media (3) and select how many columns you will use
9. Go to solvent tips (1) and sample tips (2) and check tips are filled as displayed on screen. If necessary, add or delete missing columns (or add tips)
10. Click “run method”
11. Monitor that the extraction procedure initiated correctly
12. At the end of the extraction, transfer the eluted samples (i.e. the MF fraction) to glass tubes, rinse the collection tube or the collecting well once with MeOH and add to the sample
13. Samples are ready for drying on Turbovap

3.3.3 Solvent Evaporation

1. Switch the TurboVap Evaporator on
2. Ensure that the water bath is set to 37 °C
3. Turn nitrogen feed on and set the flow rate to no more than 15 psi
4. Place 10mL conical borosilicate tubes containing the methylformate fraction or methanol fraction obtained during solid phase extraction in the TurboVap
5. Ensure that the lid is closed
6. When the solvent is more than 95% evaporated rinse the walls of the tube using methylformate
7. When the solvent is more than 95% evaporated rinse the walls of the tube using methanol
8. When the solvent is completely evaporated, add 40µl of methanol/water (1:1)
9. Centrifuge the tube at 2000 × g for 2 min
10. Carefully transfer the supernatant to an injection vial insert

11. Place the insert in a clearly labelled 1.5 mL eppendorf tube
12. Centrifuge the insert at 10000 × *g* for no more than 2 min
13. Transfer the supernatant to an injection vial insert and place the insert in a clearly labelled sample vial.
14. Samples are now ready for LC-MS-MS profiling.

3.4 Chromatography

1. Using an Poroshell 120 EC-18 (4.6 mm × 100 mm × 2.7 µm) and water containing 0.01% acetic acid as solvent A, and methanol containing 0.01% acetic acid as solvent B, the following gradient should be used to chromatographically separate the mediators from their isomers and pathway markers
2. Column temperature should be set at 50 °C
3. Equilibrate the column with mobile phase at 80:20 (A:B)
4. This should be ramped to 50:50 (A:B) over 12 seconds
5. The gradient should be maintained for 2 minutes
6. Then to 80:20 over the subsequent 9 min
7. This should be maintained for the next 3.5 min
8. Then ramped to 98:2 (A:B)
9. Finally maintain this for 5.4 min to wash the column
10. The follow rate should be maintained at 0.5 mL/min throughout the experiment

3.5 Data analysis using Analyst Quantitate tool

3.5.1 Setting up a quantitation method

1. In Analyst Click on Build Quantitation Method
2. Select a file that contains a standard mix
3. Assign which transitions correspond to the internal standards

4. Assign the internal standards that will be used for identification and quantitation of each molecule.
5. In the Integrate tab select a retention time window of not more than 5 seconds
6. Save the method

3.5.2 Analysing the data

1. Click the quantitation wizard and follow the instructions to load your samples and the appropriate analysis method obtained as detailed above
2. Start by integrating the deuterium-labelled internal standards making a note of any drifts from the expected retention times
3. If retention-time drifts are observed correct the expected retention times for each of the analyte according to the retention time drift observed in the respective internal standards being used for identification (e.g. if d_5-LXA_4 is observed to elute 2 seconds after the anticipated retention time then LXA_4 expected retention time will also shift by 2 seconds)
4. After determining the expected retention times for each of the samples integrate each of the mediators. Here all peaks that are clearly visible even if not more than 3 times higher than baseline should be integrated.
5. After integrating all the peaks in explore mode search for MS-MS spectra for molecules where a peak was obtained in at least 1 transition in the same region of the chromatogram where the peak was recorded.
6. Compare the tandem mass spectrum obtained in the sample with that obtained from the synthetic/authentic compound matching at least 6 ions in the MS-MS spectrum, with one of the ions being derived from a backbone break. These can be compared and matched to the Spectra Book available for all of these mediators and pathways on the Serhan lab website.

7. For those molecules where the MS-MS spectrum is a positive match proceed to quantitation using a standard curve constructed using either synthetic or authentic standard mixes with concentrations determined as detailed above.
8. For molecules where standards are not available, molecules that carry similar physical properties may be used.
9. If an MS-MS spectrum is not obtained for a molecule or group of molecules and additional sample is available this could be re-run looking specifically for the molecule(s) where a spectrum was not obtained.
10. If no additional sample is available then the identification criteria are not fulfilled.

4. Notes

1. Concentrated HCl and acetic acid should be handled in a fumehood. Gloves and eye protection need to be wore at all times when handling these acids. Also always add the acid to water to avoid injury..
2. All standard stocks should be stored under nitrogen, shielded from light and at -20^{o}C for short-term storage and -80^{o}C for long-term storage.
3. Each time the stock is used, purge the vial with a gentle stream of nitrogen very briefly before closing it to prevent oxidative degradation.
4. All solvents are flammable, they should be handled with care in a fumehood and kept away from open flames
5. All compound stocks should be handled using zero dead volume Hamilton syringes
6. Ensure that the Hamilton syringes are appropriately cleaned using isopropanol and methanol; this should be done by aliquoting at least 20 syringe volumes with each solvent.

7. Only use nitrogen or an inert gas to evaporate solvents, do not use air since this will lead to mediator oxidation.

8 Upon solvent evaporation and suspension of samples in water/methanol samples need to be profiled within 24h to avoid isomerization of the mediators. At all times samples should be kept at 4^{o}C and not frozen to maximise mediator integrity.

9. DO NOT DMSO at any stage since SPM are sensitive to isomerization and oxidation in this solvent.

Acknowledgements

This work was supported by funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (Grant number: 677542), a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society (Grant number: 107613/Z/15/Z) and the Barts Charity (Grant number: MGU0343). CN Serhan is supported by the National Institutes of Health, USA Grant Number P01GM095467.

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Tables

Table 1 List of deuterated internal standards, their complete stereochemistries and source.

Abbreviation	***Trivial name***	***Full stereochemistry***	***Source***
5S-HETE-d_8	Deuterium-labelled 5S-HETE	5S-hydroxy-eicosa-6E,8Z,11Z,14Z-tetraenoic-5,6,8,9,11,12,14,15-d_8 acid	Cayman Chemicals
LTB_4-d_4	Deuterium-labelled leukotriene B_4	5S,12R-dihydroxy-eicosa-6Z,8E,10E,14Z-tetraenoic-6,7,14,15-d_4 acid	Cayman Chemicals
LXA_4-d_5	Deuterium-labelled lipoxin A_4	5S,6R,15S-trihydroxy-eicosa-7E,9E,11Z,13E-tetraenoic-19,19,20,20,20-d5 acid	Cayman Chemicals
RvD2-d_5	Deuterium-labelled resolvin D2	7S,16R,17S-trihydroxy-docosa-4Z,8E,10Z,12E,14E,19Z-hexaenoic-21,21,22,22,22-d_5 acid	Cayman Chemicals
PGE_2-d_4	Deuterium-labelled prostaglandin E_2	9-oxo-11α,15S-dihydroxy-prosta-5Z,13E-dien-1-oic-3,3,4,4-d_4 acid	Cayman Chemicals

Table 2 List of arachidonic acid metabolome, their complete stereochemistries and source.

Abbreviation	***Trivial name***	***Full stereochemistry***	***Source***
AA	Arachidonic acid	eicosa-5Z,8Z,11Z,14Z-tetraenoic acid	Cayman Chemicals
PGD_2	Prostaglandin D_2	9α,15S-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic acid	Cayman Chemicals
PGE_2	Prostaglandin E_2	9-oxo-11α,15S-dihydroxy-prosta-5Z,13E-dien-1-oic acid	Cayman Chemicals
PGF_{2a}	Prostaglandin F_{2a}	9α,11α,15S-trihydroxy-prosta-5Z,13E-dien-1-oic acid	Cayman Chemicals
TxB_2	Thromboxane B_2	9α,11,15S-trihydroxy-thromba-5Z,13E-dien-1-oic acid	Cayman Chemicals
LTB_4	Leukotriene B_4	5S,12R-dihydroxy-eicosa-6Z,8E,10E,14Z-tetraenoic acid	Cayman Chemicals
LXA_4	Lipoxin A_4	5S,6R,15S-trihydroxy-eicosa-7E,9E,11Z,13E-tetraenoic acid	Cayman Chemicals
LXB_4	Lipoxin B_4	5S,14R,15S-trihydroxy-eicosa-6E,8Z,10E,12E-tetraenoic acid	Cayman Chemicals
15-HETE		15S-hydroxy-eicosa-5Z,8Z,11Z,13E-tetraenoic acid	Cayman Chemicals
12-HETE		12-hydroxy-eicosa-5Z,8Z,10E,14Z-tetraenoic acid	Cayman Chemicals
5-HETE		5S-hydroxy-eicosa-6E,8Z,11Z,14Z-tetraenoic acid	Cayman Chemicals
Δ6-trans-LTB_4	Δ6-trans-leukotriene B_4	5S,12R-dihydroxy-eicosa-6E,8E,10E,14Z-tetraenoic acid	Cayman Chemicals
5S,12S-diHETE		5S,12S-dihydroxy-eicosa-6E,8E,10E,14Z-tetraenoic acid	Biogenic synthesis

Table 3 List of eicosapentaenoic acid metabolome, their complete stereochemistries and source.

Abbreviation	***Trivial name***	***Full stereochemistry***	
EPA		eicosa-5Z,8Z,11Z,14Z,17Z-pentaenoic acid	Cayman Chemicals
RvE1	Resolvin E1	5*S*,12*R*,18*R*-trihydroxy-eicosa-6Z,8E,10E,14Z,16E-pentaenoic acid	Cayman Chemicals
RvE2	Resolvin E2	5S,18R-dihydroxy-eicosa-6Z,8E,11E,14E,16Z-pentaenoic acid	Biogenic synthesis
RvE3	Resolvin E3	17*R*,18*R/S*-dihydroxy-eicosa-5Z,8Z,11Z,13E,15E-pentaenoic acid	Custom Synthesis (Dr Makoto Arita, Riken Institute Japan)
18-HEPE		18-hydroxy-eicosa-5Z,8Z,11Z,14Z,16E-pentaenoic acid	Cayman Chemicals
15-HEPE		15-hydroxy-eicosa-5Z,8Z,11Z,13E,17Z-pentaenoic acid	Cayman Chemicals
12-HEPE		12-hydroxy-eicosa-5Z,8Z,10E,14Z,17Z-pentaenoic acid	Cayman Chemicals
5-HEPE		5-hydroxy-eicosa-6E,8Z,11Z,14Z,17Z-pentaenoic acid	Cayman Chemicals

Table 4 List of docosahexaenoic acid metabolome, their complete stereochemistries and source.

Abbreviation	***Trivial name***	***Full stereochemistry***	
DHA		docosa-4Z,7Z,10Z,13Z,16Z,19Z-hexaenoic acid	Cayman Chemicals
RvD1	Resolvin D1	7*S*,8*R*,17*S*-trihydroxy-docosa-4Z,9E,11E,13Z,15E,19Z-hexaenoic acid	Cayman Chemicals
RvD2	Resolvin D2	7S,16R,17S-trihydroxy-docosa-4Z,8E,10Z,12E,14E,19Z-hexaenoic acid	Cayman Chemicals
RvD3	Resolvin D3	4*S*,7*R*,17*S*-trihydroxy-docosa-5Z,7E,9E,13Z,15E,19Z-hexaenoic acid	Cayman Chemicals
RvD4	Resolvin D4	4S,5R,17S-trihydroxy-docosa-6E,8E,10Z,13Z,15E,19Z-hexaenoic acid	Custom Synthesis (Dr Charles Serhan
RvD5	Resolvin D5	7*S*,17*S*-dihydroxy-docosa-4Z,8E,10Z,13Z,15E,19Z-hexaenoic acid	Cayman Chemicals
RvD6	Resolvin D6	4S,17S-dihydroxy-docosa-5E,7Z,10Z,13Z,15E,19Z-hexaenoic acid	Biogenic Synthesis
MaR1	Maresin 1	7*R*,14*S*-dihydroxy-docosa-4Z,8*E*,10*E*,12*Z*,16*Z*,19*Z*-hexaenoic acid	Cayman Chemicals
4S,14S-diHDHA		4S,14S-dihydroxy-docosa-5Z,7E,10E,12Z,16E,19E-hexaenoic acid	Biogenic Synthesis
7S,14S-diHDHA		7S,14S-dihydroxy-docosa-4Z,8E,10E,12Z,16E,19E-hexaenoic acid	Biogenic Synthesis
PD1	Protectin D1	10R,17S-dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid	Custom Synthesis (Dr Charles Serhan)
10S,17S-diHDHA	Protectin Dx	10*S,*17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-hexaenoic acid	Cayman Chemicals
22-OH-PD1	22-OH-Protectin D1	10R,17S,20-trihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid	Custom Synthesis (Dr Trond V. Hansen, University of Oslo)
17-HDHA		17-hydroxy-docosa-4Z,7Z,10Z,13Z,15E,19Z-hexaenoic acid	Cayman Chemicals
14-HDHA		14S-hydroxy-docosa-4Z,7Z,10Z,12E,16Z,19Z-hexaenoic acid	Cayman Chemicals
13-HDHA		13-hydroxy-docosa-4Z,7Z,10Z,14E,16Z,19Z-hexaenoic acid	Cayman Chemicals
7-HDHA		7-hydroxy-docosa-4Z,8E,10Z,13Z,16Z,19Z-hexaenoic acid	Cayman Chemicals
4-HDHA		4-hydroxy-docosa-5E,7Z,10Z,13Z,16Z,19Z-hexaenoic acid	Cayman Chemicals

Table 4 List of aspirin triggered mediators, their complete stereochemistries and source.

Abbreviation	***Trivial name***	***Full stereochemistry***	
AT-LXA_4	Aspirin-triggered lipoxin A_4	5S,6R,15R-trihydroxy-eicosa-7E,9E,11Z,13E-tetraenoic acid	Cayman Chemicals
AT-LXB_4	Aspirin-triggered lipoxin B_4	5S,14R,15R-trihydroxy-eicosa-6E,8Z,10E,12E-tetraenoic acid	Cayman Chemicals
AT-RvD1	Aspirin-triggered Resolvin D1	7S,8R,17R-trihydroxy-docosa-4Z,9E,11E,13Z,15E,19Z-hexaenoic acid	Cayman Chemicals
AT-RvD3	Aspirin-triggered Resolvin D3	4*S*,7*R*,17*R*-trihydroxy-docosa-5Z,7E,9E,13Z,15E,19Z-hexaenoic acid	Custom Synthesis (Dr Charles Serhan)
AT-PD1	Aspirin-triggered Protectin D1	10R,17R-dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid	Custom Synthesis (Dr Charles Serhan)

Table 5 Extinction co-efficient and λ_{max}^{MEOH} for distinct conjugated double bond systems

Double bond system	**Extinction Co-efficient**	λ_{max}^{MEOH}
Conjugated diene (e.g:monohydroxy acids)	25,000	235
Two conjugated dienes (e.g. RvD5)	25,000	240
Diene-triene conjugated system (e.g. RvE1)	40,000	271
Conjugated Triene (e.g. PD1)	40,000	269
Conjugated Tetraene (e.g. RvD1)	50,000	301

Figure Legends

Figure 1. Sample preparation. Tissues from various origins (human or animal tissues or fluids, mice, planaria, cell culture, etc.) should be homogenized in ice-cold MeOH containing deuterated internal standards. Samples should always be kept on ice to prevent mediator isomerization. For biological fluids 4 volumes of methanol should be added to the samples. Homogenized tissues and biological fluids should then be kept at -20°C for 45 min to allow for protein precipitation. Samples should then be centrifuged, supernatant collected and acidified to pH 3.5 with HCl prior to solid phase extraction. The eluting fraction containing the bioactive lipid mediators is dried under a gentle flux of nitrogen (N_2) and resuspended in phase prior injection. MeOH, methanol; H_2O, water; MF, methyl formate; N_2, nitrogen.

Figure 2. Schematic of instrument setup for mediator identification. Samples in phase are injected using a HPLC-MS-MS system, no more than 40µl should be injected. LC, liquid chromatography; RP, reverse phase; MeOH, methanol; ESI, electron spay ionization; m/z, mass to charge ratio; MRM, multiple reaction monitoring; EPI, enhanced product ion

Figure 3. Lipid mediator identification and quantification. Identification of bioactive LM is performed by matching retention time (RT) and at least 6 diagnostic ions from the MS-MS spectrum with those of synthetic or authentic standards for each mediator. Quantitation is achieved using linear regression curves that are constructed using synthetic or authentic standards for each mediator of interest. Interrelationship(s) for identified mediators within each condition and between different conditions is further

investigated using multivariate analysis. LM, lipid mediator; RT, retention time; AUC, area under the curve; PCA, principal component analysis; PLS-DA, partial least square-discriminant analysis; OPLS-DA, orthogonal partial least square-discriminant analysis