Chapter 3

Determination of 2-Arachidonoylglycerol by μSPE-LC-MS/MS

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Abstract

LC-MS/MS is a powerful analytical technique that provides unequivocal identification and reliable quantification of the analytes, using Selected Reaction Monitoring or Multi Reaction Monitoring acquisition mode.

2-Arachidonoylglycerol (2-AG) is the most abundant endocannabinoid, which plays a major role in a wide variety of physiological and pathological processes. Analysis of 2-AG by means of LC-MS/MS allows the detection of very low concentrations in biological samples. Here, we describe how to determine 2-AG levels in tiny samples of tissues and plasma through LC-MS/MS, by using very quick and easy to perform extraction procedures, with reduced solvent consumption.

Key words 2-AG, μSPE, LC-MS/MS, Plasma, Tissue

1 Introduction

High-performance liquid chromatography (HPLC) is a well-established separation technique that is able to solve numerous analytical problems. Several developments have been done recently to improve HPLC performance, the most important possibly being Ultra High Performance Liquid Chromatography (UHPLC or UPLC) [1, 2]. Yet, due to the cost of UPLC hardware, chromatographic research addressed to improve HPLC performance in terms of faster separation and efficiency. Thus, when UPLC is not available, alternative devices and technologies have been developed for fast chromatography: high-temperature liquid chromatography (HTLC), and use of monolithic supports or of column with superficially porous packing materials based on silica particles with non-porous cores [3]. The latter solution is very interesting, because it allows obtaining high efficiency with common HPLC devices.

Liquid chromatography–mass spectrometry or tandem mass spectrometry (LC-MS or LC-MS/MS) is largely replacing gas chromatography (GC)-MS in most biomedical applications, as

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they do not require derivatization steps and are capable of simultaneous determination in a single analysis with reduced sample pretreatment [4].

Moreover, MS/MS is considered necessary to provide unequivocal identification and quantification of analytes, due to the high selectivity of the MS detector [5]. The quadrupole is the most common mass analyzer, due to its cheapness and high performance allowed by high efficiency transmission. The first implementation of a two-dimensional MS (MS/MS or MS^2) was obtained with a triple quadrupole mass analyzer that is nowadays the most common device. The first and third (Q1 and Q3) quadrupoles are separative, and the second (Q2) is usually a collision cell, where the ions can be fragmented, as depicted in Fig. 1.

MS^2 enables the reduction of background noise and increase in selectivity, two features that also allow different types of experiments: Full Scan (FS), Selected Ion Monitoring (SIM), Product Ion Scan (PIS), Precursor Ion Scan, Selected Reaction Monitoring or Multi Reaction Monitoring (SRM or MRM), the latter being most widely used for the confirmatory analysis of target molecules.

LC and MS coupling is not straightforward, as chromatographic separation occurs with neutral molecules in high operating pressure solution, while MS responds to ions in the gas phase and requires deep vacuum [6].

The most widely used technique is electrospray ionization (ESI), which is suitable for the analysis of medium to highly polar compounds. Atmospheric pressure chemical ionization (APCI) is more efficient for nonpolar (hydrophobic) analytes, which do not readily form ions in solution [7]. Instead, thermally labile compounds may decompose in the ionization source, since APCI requires a heated nebulizer [7].

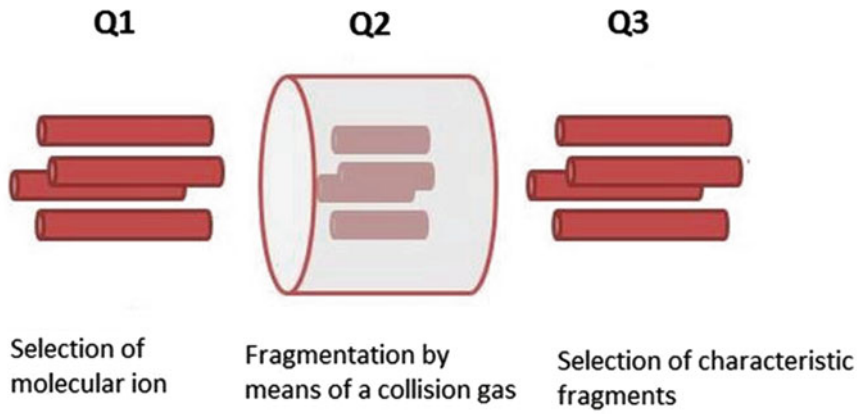

Fig. 1 Schematic description of a triple quadrupole mass spectrometer: first quadrupole (Q1), second quadrupole (Q2), which acts as a collision cell, and third quadrupole (Q3)

2-Arachidonoylglycerol (2-AG) is the most abundant endocannabinoid, identified for the first time in brain tissues [8]. 2-AG plays a major role in a wide variety of physiological and pathological processes [9]. The measurement of circulating levels of 2-AG in several biological matrices, including also fluids such as blood [10–12] and seminal plasma [13], is an essential step to determine both "signaling" and "metabolic-intermediate" levels of this endocannabinoid [14]. Despite great improvements in instrumentation, the quantitation of 2-AG remains an analytical challenge, also because of its instability that makes it easily isomerize into 1-AG [15].

2 Materials

2-AG and [$^2H_8$]-2-AG standard (0.26 mM, 100 μg/ml in acetonitrile).

Acetone, acetonitrile, chloroform, formic acid, and methanol are of RS-Plus grade.

Ultrapure water is produced by a Milli-Q Plus apparatus.

OMIX C_{18} tips.

Kinetex C18-XB chromatographic column (100 × 2.1 mm) packed with 2.6 μm average diameter core-shell particles, and provided with a guard column.

2.1 Specimens Collection

Tissues must be snap-frozen in liquid nitrogen after collection (*see* **Note 1**) and stored at −80 °C (*see* **Note 2**).

2.1.1 Tissue

2.1.2 Plasma

1. Collect blood in a Vacutainer tube with sodium citrate.
2. Centrifuge blood in the collection tube for 15 min at 150 × *g* with brake off switch.
3. Remove the tube from the centrifuge.
4. Transfer plasma (top layer) to a 15 ml centrifuge tube. Be careful not to aspirate cells from the buffy coat (cellular) layer.
5. Centrifuge plasma in the 15 ml centrifuge tube for 15 min at 1000 × *g*.
6. Transfer plasma into a 8 ml glass vial and store at −80 °C (*see* **Note 2**).

3 Methods

3.1 Extraction from Tissue

1. Warm at room temperature and vortex the [$^2H_8$]-2-AG standard (stored at −80 °C).
2. Prepare a solution of internal standard (IS) in ice-cold methanol, in order to add 50 pmoles of [$^2H_8$]-2-AG per sample.

3. Add 1 ml IS solution *per* 8 ml glass vial and keep the vial on ice.
4. Weight the frozen tissue and put it in the vial containing the IS solution.
5. Homogenize the tissue with an Ultra Turrax T25, keeping the vial on ice (*see* **Note 3**).
6. Add 2 ml chloroform per vial with vortexing.
7. Add 1 ml water per vial with vortexing.
8. Centrifuge the mixture at 2000 × *g* at 4 °C for 10 min.
9. Collect the lower organic phase using a Pasteur pipette, and discard the upper aqueous layer and the protein disk.
10. Evaporate the solvent to dryness in the vial under a gentle nitrogen stream.
11. Plug a Pasteur pipette 5–3/4″ length with glass wool.
12. Prepare a suspension of Silica Gel G (60-Å 230–400 Mesh ASTM) in chloroform (1:1, v/v) and load 1 ml of this suspension onto the Pasteur pipette from **step 11**.
13. Dissolve the dried lipids into 1 ml chloroform.
14. Load the sample onto the Silica G gel column.
15. Wash the vial with 1 ml of chloroform and repeat **step 14**.
16. Elute the analyte from the column with 1 ml of chloroform/methanol (9:1, v/v) and recover the eluate. Repeat **step 16**.
17. The collected eluate is dried under a gentle nitrogen stream.
18. Dissolve the dried lipids into 100 μl methanol and transfer the mixture to 1 ml glass vial with 0.1 ml conical glass insert.
19. The vial is transferred into the autosampler for the LC-MS/MS analysis.

3.2 Extraction from Plasma

1. Warm at room temperature and vortex the [$^2H_8$]-2-AG standard stored at −80 °C.
2. Prepare a solution of IS in 200 mM formic acid in ice-cold methanol, in order to add 2 pmoles of [$^2H_8$]-2-AG per sample.
3. Mix 100 μl of plasma with 100 μl of IS solution.
4. Sonicate the sample in an ultrasonic bath for 6 min at room temperature.
5. Centrifuge at 17,500 × *g* for 6 min at 4 °C.
6. Recover the upper phase and transfer into a 1 ml glass vial with 0.1 ml conical glass insert.
7. Condition the μSPE tip by flushing four times a 1:1 ultrapure water/acetonitrile solution and four times a 1:1 ultrapure

water/200 mM formic acid in methanol using an automatic propipette.

8. Load the sample by inserting the OMIX C_{18} tip into the vial containing the sample, and perform five load/release cycles.
9. Wash with 100 μl of a 9:1 (v/v) water/methanol solution (five load/release cycles).
10. Elute the analytes with 50 μl of 10 mM formic acid in methanol. Repeat this step five times, performing five load/release cycles for each step.
11. The collected eluate is transferred into 1 ml glass vial with 0.1 ml conical glass insert.
12. The vial is transferred into the autosampler for the LC-MS/MS analysis.

3.3 LC-MS/MS Analysis

The chromatographic run is carried out by means of a Series 200 system with 2 μ-LC pumps and autosampler from Perkin Elmer (Norwalk, CT, USA). The HPLC system is coupled to an API 2000 triple quadrupole mass spectrometer from ABSciex (Toronto, ON, Canada), equipped with a TurboIonSpray source.

The mobile phases are water (A) and 2.5 mM formic acid in methanol (B), at a flow rate of 0.4 ml min^{-1}. Only 0.1 ml min^{-1} is driven into the ion source by means of a 1:4 split. A gradient elution is applied, so that the organic phase increases from 80 to 84 % in 3 min and then to 100 % in 1 min. After 3 min at 100 % of organic phase, the column is led to the original ratio of 20 % B and 80 % A within 3 min, in order to enable equilibration of the column.

A timed switch valve (10-PORT Valco valve connected to the instrument) drives the effluent to the source only from 3 to 6.5 min. The resulting total run-time is 10 min.

All the analytes are detected in positive ionization mode with a capillary voltage of 5000 V and nebulizer gas (air) at 90 psi, while the turbo gas (nitrogen) is at 40 psi and 400 °C. For each analyte, two MRM transitions are selected. All sources and instrument parameters for the monitored analytes are tuned by injecting standard solutions at a concentration of 100 ng ml^{-1} (containing 10 mM of formic acid) at 10 μl min^{-1} by a syringe pump.

Peak areas for the selected ions are determined using ABSciex package Multiview 1.4 and quantitation is performed by the IS method. The selected transitions, together with the main LC-MS/MS parameters, are reported in Table 1 (*see* **Note 4**).

The quantification of 2-AG is accomplished by the use of deuterium-labeled IS *vs* a calibration curve in methanol acidified with 10 mM formic acid, in a 2.64–528 nM range (*see* **Note 5**).

A chromatogram showing the separation between 2-AG and 1-AG, obtained with the above described experimental conditions, is reported in Fig. 2.

Table 1
LC-MS/MS parameters for 2-AG and its IS

Analyte	RT (min)	Q1 (amu)	DP (V)	FP (V)	EP (V)	Q3 (amu)	CE (V)	CXP (V)
2-AG	4.2	379.1	21	400	7	287.2	21	15
						269.1	26	14
[$^2H_8$]-2-AG	4.2	387.2	21	400	7	295.0	21	15

RT retention time, *Q1* precursor ion mass, *DP* declustering potential, *FP* focusing potential, *EP* entrance potential, *Q3* product ion mass, *CE* collision energy, *CXP* cell exit potential

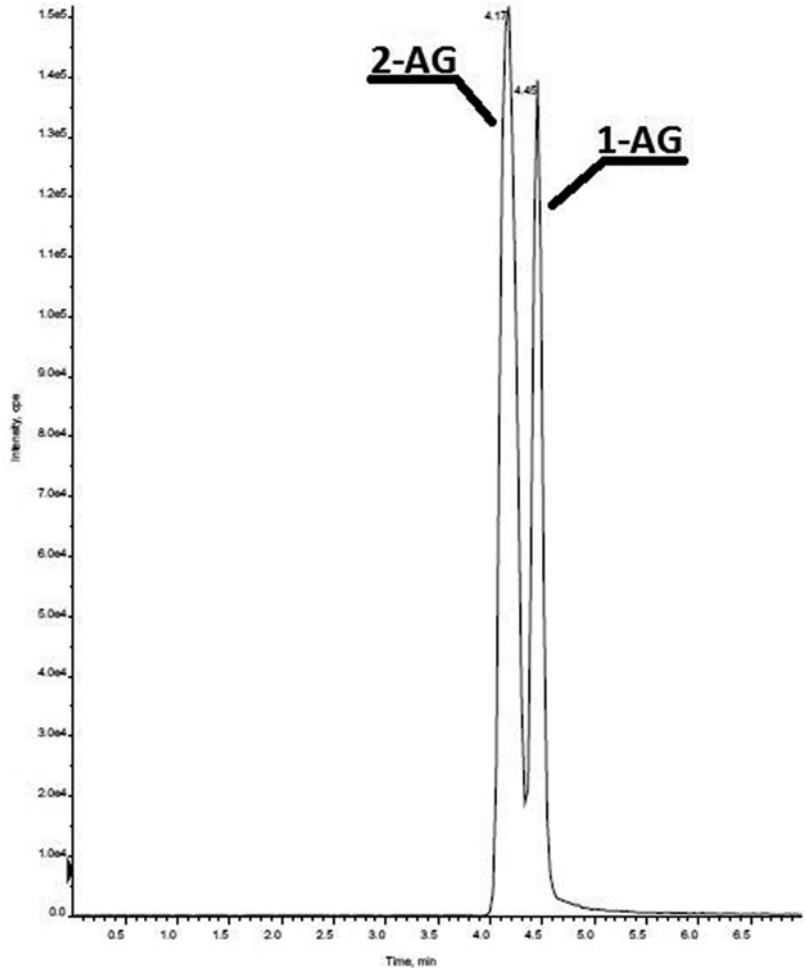

Fig. 2 Chromatogram of 2-AG and 1-AG obtained by LC-MS/MS in MRM acquisition mode

4 Notes

1. Use only Teflon caps, glass vials, and glass pipettes.
2. Biological samples must be snap-frozen at –80 °C immediately after collection.
3. It may be useful to normalize the amount of lipids to the protein content of the samples (cells or tissues). Before starting the extraction procedure, take 20 µl aliquots from the homogenates and determine protein concentration by using a Bradford protein concentration assay.
4. Perform quantitative analysis by selecting the $[M+H]^+$ adduct: 2-AG may form $[M+Na]^+$ adducts which do not lead to any fragmentation. It is important to verify the ratio between the $[M+H]^+$ adduct and the $[M+Na]^+$ adduct, to avoid dramatic loss in sensitivity.
5. Plot calibration curves as the peak area ratio of the analyte over its respective IS against the nominal concentration of the calibrator, by least square linear regression.

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