TITLE:

Synthesis of a Deuterated Standard for the Quantification of 2-Arachidonoylglycerol in *C. elegans*

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SUMMARY

This work describes a robust and straightforward method to detect and quantify the endocannabinoid 2-arachidonoylglycerol (2-AG) in *C. elegans*. An analytical deuterated standard was prepared and used for the quantification of 2-AG by isotopic dilution and liquid chromatography-electrospray Ionization-tandem mass spectrometry (LC-ESI-MS/MS).

ABSTRACT:

We present a method to easily prepare an analytical standard to analyze 2-arachidonoyl glycerol (2-AG) qualitatively and quantitatively by liquid chromatography-electrospray Ionization-tandem mass spectrometry (LC-ESI-MS/MS). Endocannabinoids are conserved lipid mediators that regulate multiple biological processes in a variety of organisms. In *C. elegans*, 2-AG has been found to display different roles including modulation of dauer formation and cholesterol metabolism. In this report, we describe a method to overcome the difficulties associated with the cost and stability of the deuterated standards required for 2-AG

quantification. The three-step procedure for the synthesis of the standard is simple and can be performed in any laboratory without the need of organic synthesis expertise or special equipment. In addition, we describe a modification of Folch´s method to extract the deuterated standard from *C. elegans* cultures. Finally, we detail a quantitative analysis method to detect 2-AG using the stable isotopically labeled analog 1-AG-d_5, which provides reliable results in a fast-chromatographic run. The present procedure will be useful for studying the multiple roles of 2-AG in *C. elegans* being also applicable to the study of the metabolite in different organisms.

INTRODUCTION:

Endocannabinoids regulate multiple biological processes in a variety of organisms and are conserved lipid mediators [1]. The first discovered and best-characterized endocannabinoids are anandamide (arachidonoylethanolamide, AEA) and 2-arachidonoyl glycerol (2-AG). Endocannabinoids play many critical roles including brain reward systems, drug addiction, memory, mood and metabolic processes[2]. AEA and 2-AG are only synthesized when needed and have a short life span being degraded through transport protein reuptake and hydrolysis[3].

The use of animal model like *C. elegans* has become important to study the large variety of biological processes including apoptosis, cell signaling, cell cycle, cell polarity, gene regulation, metabolism, ageing and sex determination[4,5]. Additionally, *C. elegans* is an excellent model for studying the physiological roles of polyunsaturated fatty acids (PUFAs). AEA has been identified in *C. elegans,* being reduced under dietary restriction[6]. This deficiency extends the lifespan of the nematode through a dietary restriction mechanism that can be suppressed by supplementation with the endocannabinoid. Recently, we discovered that 2-AG and AEA play a fundamental role in the regulation of cholesterol trafficking in *C. elegans*[7]. More importantly, we determined that supplementation with exogenous 2-AG can rescue the dauer arrest caused by the impaired cholesterol trafficking of Niemann Pick C1 *C. elegans* mutants.

To gain a better understanding of its relationship with cholesterol trafficking and other biological processes in the nematode like monoaminergic signaling, nociception and locomotion, it is crucial to study this endogenous metabolite and how it is affected by the environmental and dietary conditions [8-13]. Therefore, it became imperative to design and optimize a method to detect and quantify endogenous 2-AG in *C. elegans* that would be simple to use for scientists from different fields that need to study the nematode's behavior in relation to this endocannabinoid.

In 2008, Lethonen and his coworkers succeeded in identifying 2-AG and AEA in *C. elegans* by a LC-MS analytical method[14] and in 2011 he managed to expand his technique to other endocannabinoids[15]. More recent work has shown other analytical methods to detect and quantify endocannabinoids in *C. elegans* involving mass spectrometry and GC-MS[16-18] and it has also been reported that analytical methods of the sort can also be expanded to other study models[19].

Previously reported analytical methods to quantify 2-AG in biological samples usually involve the use of deuterated standards that are commercially acquired and require having them

available for purchase[20,21]. Many analytical standards for LC-MS/MS quantification of endocannabinoids are commercially available from different providers. Nevertheless, they are expensive and due to the presence of multiple double bonds, are sensitive and get oxidized over time. The most common versions of the standards are based on the octa-deuterated arachidonic acid, being suitable for quantification by isotope dilution LC-MS/MS[14,22]. Also, most of these standards are substituted in position 2 of the glycerol being very unstable under most conditions since they are prone to acyl migration[19,23].

To overcome the difficulties associated with the cost and stability of the deuterated standards to quantify 2-AG, we used a convenient and simple method to prepare an analytical standard based on glycerol-d_5. The sequence to prepare the penta-deuterated standard requires a three steps procedure that results in the standard 1-AG-d_5, which is stable and does not undergo acyl migration, a main issue when aiming to synthesize 2-monoacylglycerols.

The main objective of this work is to show a simple and reproducible method to study 2-AG in *C. elegans*, from the synthesis of the analytical deuterated standard, to the preparation and extraction of the nematode samples and finally the analysis by LC-MS/MS (**Figure 1**). This synthetic procedure is achievable without the need of sophisticated organic synthesis knowledge or special equipment, being suitable for scientists from different fields studying *C. elegans* behavior under endocannabinoids influence. The method is also expandable to other study models making it useful for different targets.

The standard, prepared as reported here, has been applied to successfully develop a fast and reliable chromatographic method that allowed an effective detection and quantification of 2-AG in a reproducible manner.

PROTOCOL:

1. 1-AG-d_5 preparation

NOTE: For obtaining 1-AG-d_5 as a deuterated internal standard for quantification assays, follow a three-step protocol as detailed below.

1.1. Differential protection step.

1.1.1. In order to only protect primary alcohols, first, add 38 mg of glycerol-d_8 to a 10 mL reaction tube using a Pasteur pipette and add a magnetic stirrer.

1.1.2. Add 5 mL of anhydrous dichloromethane (DCM) using a 5 mL Hamilton syringe, and fill the tube with dry N_2 to give an inert atmosphere.

1.1.3. Prepare a bath using a shallow Dewar flask filled with distilled ethyl acetate.

1.1.4. Fit the hermetically closed reaction tube inside the bath and cool it by slowly adding liquid N_2 to the ethyl acetate until the solvent is frozen.

CAUTION: Liquid violently boils at room temperature and might cause severe burns if in contact with eyes and skin.

1.1.5. Add 54 mg of anhydrous collidine using a Hamilton syringe.

CAUTION: Collidine is volatile and has a very strong and unpleasant smell.

1.1.6. Add 70 mg of tert-butyldimethylsilyl chloride and stir everything for 3 h at -78 °C on a magnetic stirrer.

1.1.7. After 3 h, leave the reaction to warm at room temperature and keep the stirring for additional 12 h.

1.1.8. Add 2 mL of brine to quench the reaction.

1.1.9. Extract the solution with 2 mL of distilled dichloromethane, 3 times using a separating funnel, saving the organic extract each time.

1.1.10. Combine the three organic extracts and dry over sodium sulfate.

1.1.11. Evaporate the dichloromethane under reduced pressure in a vacuum rotary evaporator carefully to avoid solvent projections.

1.1.12. Purify the crude mixture by column chromatography using silica gel as the stationary phase and a 10% increasing hexane/ethyl acetate gradient starting from 100% hexane and finishing with 100% ethyl acetate.

1.1.13. Combine the product-containing fractions and remove the solvent under reduced pressure in a vacuum rotary evaporator to obtain the pure 1-O,3-O-bis-(TBDMS) glycerol-d_5 as a colorless liquid.

1.2. Esterification step.

1.2.1. Add 10 mg of the 1-O,3-O-bis(TBDMS)-glycerol-d_5 previously synthesized to a 10 mL reaction tube using a Pasteur pipette and add a magnetic stirrer.

1.2.2. Add 2 mL of anhydrous dichloromethane using a 5 mL Hamilton syringe, and fill the tube with dry N_2 to provide an inert atmosphere.

1.2.3. Cool the solution to 0 °C using an ice bath.

1.2.4. Add 36 mg of arachidonic acid using a multi-volume adjustable micropipette and stir.

1.2.5. Add 15 mg of 4-dimethylaminopyridine and stir.

1.2.6. Add 15 mg of N,N'-diisopropylcarbodiimide using a multi-volume adjustable micropipette and stir.

1.2.7. Let the mixture react at 0 °C for 3 h.

1.2.8. After 3 h, leave the reaction to warm at room temperature and keep the stirring on for an additional 12 h.

1.2.9. Add 2 mL of water to quench the reaction.

1.2.10. Extract the organic solution with 2 mL of distilled dichloromethane (DCM), 3 times using a separating funnel.

1.2.11. Place the three organic extracts in the same tube and dry over sodium sulfate.

1.2.12. Evaporate the dichloromethane under reduced pressure in a vacuum rotary evaporator carefully to avoid solvent projections.

1.2.13. Purify the crude mixture by column chromatography using silica gel as the stationary phase and a 10% increasing hexane/ ethyl acetate gradient starting from 100% hexane and finishing with 50% hexane/50% ethyl acetate.

1.2.14. Combine the product-containing fractions and remove the solvent under reduced pressure in a vacuum rotary evaporator to obtain the pure 1-O, 3-O-bis(TBDMS)-2-AG-d_5 as a yellowish liquid.

1.3. Deprotection step.

1.3.1. Add 15 mg of the 1-O,3-O-bis(TBDMS)-2-AG-d_5 previously synthesized to a 10 mL reaction tube using a Pasteur pipette and add a magnetic stirrer.

1.3.2. Add 2 mL of anhydrous THF using a 5 mL Hamilton syringe, and fill the tube with dry N_2 to give an inert atmosphere.

1.3.3. Cool the solution to 0 °C using an ice bath.

1.3.4. Add 150 µL dropwise of a 1 M solution of tetrabutylammonium fluoride in THF using a Hamilton syringe.

1.3.5. Let the reaction warm to room temperature and stir for 1 h.

1.3.6. After 1 h, add 2 mL of water to quench the reaction.

1.3.7. Extract the solution with 2 mL of distilled dichloromethane, 3 times using a separating funnel.

1.3.8. Combine the three organic extracts and dry over sodium sulfate.

1.3.9. Evaporate the dichloromethane under reduced pressure in a vacuum rotary evaporator to obtain the pure 1-AG-d_5 as a yellowish liquid.

1.4. Monitor all reactions by thin layer chromatography performed on silica gel 60 F_{254} pre-coated aluminum sheets. Visualize the bands under a 254 nm UV lamp, after staining with an ethanolic solution of 4-anisaldehyde.

2. Preparation of Standard Stock Solution and measuring solution

2.1. Dissolve 1 mg of the internal standard 1-AG-d_5 in 1 mL of ACN and sonicate for 1 min to get the 1000 ppm standard stock solution.

2.2. To prepare the 1000 ppb solution used for quantification in worms, first, prepare a 10ppm solution: Take 10 µL of the stock solution using a Hamilton syringe and dilute it to a final volume of 1mL by adding 990 µL of ACN.

2.3. Take 100 µL from it using a Hamilton syringe and dilute it to a final volume of 1 mL by adding 900 µL of ACN to get the 1000 ppb solution used for quantification in worms.

2.4. Sonicate for 1 min each time between steps to ensure complete solubilization. Store the solutions at -78 °C to keep the concentrations and integrity of the standards. After the standard solution is used, flow some nitrogen before closing the vial to prevent oxidation.

3. Growth and maintenance of *C. elegans*

NOTE: Seed the nematode growth medium (NGM) agar plates with *E. coli* OP50 and propagate the worms on these plates.

3.1. Mix 3 g of NaCl with 17 g of agar.

3.2. Add 2.5 g of peptone and finally, 975 mL of H_2O.

3.3. Autoclave for 50 min and then, cool the flask to 55 °C.

3.4. Mix in 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$ and 25 mL, 1 M KH_2PO_4 buffer (all of them previously autoclaved) and 1 mL of 5 mg/mL cholesterol in ethanol.

3.5. Assuring a sterile environment, dispense the NGM solution into 60 mm Petri plates, filling the plates up to 2/3 of their volume. Store the plates at 4 °C.

3.6. Streak the *E. coli* bacterium culture from a -80 °C glycerol stock onto the LB agar plate. Let it grow on the plate for overnight at 37 °C.

3.7. Pick up a single colony to inoculate 100 mL of liquid LB overnight at 37 °C with agitation. NOTE: it is not necessary to check the O.D. because this strain can reach stationary phase over this time

3.8. Bring out the stored NGM plates, remove the lid in the laminar flow hood and leave it open to allow evaporation of excess moisture from the plates.

3.9. Once the plates are dried, use a Pasteur pipette to add 100 µL of OP50 *E. coli* to the center of the plate without spreading it.

3.10. Leave the OP50 *E. coli* lawn to grow overnight at room temperature or at 37 °C for 8 h.

3.11. Add the desired number worm embryos obtained by hypochlorite treatment or "bleaching" (Step 4).

NOTE: Cool the plates to room temperature before the addition of worms.

4. Bleaching technique for synchronizing *C elegans* cultures

4.1. Seed and chunk worms onto 6 cm NGM plates.

4.2. Leave the worms growing for 2-3 days to obtain many eggs and gravid adults on the plate.

4.3. Once there are an enough eggs/adult, pour 5 mL of M9 onto the plate.

4.4. Transfer the worms to a 15 mL centrifuge tube using a glass pipette.

4.5. Centrifuge the tube for 2 min at 2000 *x g* and pellet the worms.

4.6. Suction most of the M9 avoiding disturbing the worm pellet.

4.7. Add 3 mL of bleaching solution (2:1:1, NaOH: NaOCl: H_2O).

4.8. Invert gently to mix the solution for 5 min or until the number of intact adult worms decreases.

CAUTION: Do not bleach for more than 5 min.

4.9. Centrifuge for 1 min at 2000 *x g* and suction most of the bleaching solution without disturbing the worm pellet.

4.10. Add 15 mL of M9 and mix well.

4.11. Centrifuge again at 2000 *x g* for 1 min.

4.12. Suction most of the M9.

4.13. Repeat steps 4.10-4.12 one or two more times.

4.14. Add 5 mL of fresh M9 and agitate.

4.15. Let the eggs hatch overnight with gentle rocking.

5. Worm sample preparation

5.1. Let N2 embryos obtained by bleaching procedure hatching overnight in M9 buffer (5 mL in 15 mL centrifuge tube) at 20 °C.

5.2. Harvest the synchronized L1s by centrifuging the tube 2 min at 2000 *x g*.

5.3. Wash the worms with M9 buffer one time and then quantify the number of live L1.

5.4. Seed approximately 10,000 worms into NGM plates (10 cm diameter) with 1 mL of OP50 *E. coli* (previously dried).

5.5. Incubate the plates for 48 h at 20 °C until the worms reach the L4 stage.

5.6. Harvest the worms using cold M9 buffer in a 15 mL centrifuge tube, wash them one time and then and transfer them to a 1.5 mL tube.

5.7. Pellet the worms by centrifugation at 2000 *x g* for 1 min, eliminate most of the supernatant, then immerse the tubes in liquid nitrogen and store at -80 °C.

6. Lipid extraction

6.1. Thaw on ice approximately 100 µL of frozen worm pellets belonging to N2, add 1.3 mL of methanol and sonicate the sample for 4 min.

6.2. Add 2.6 mL of chloroform, 1.3 mL of 0.5 M KCl/0.08 M H_3PO_4 to a final ratio of 1:2:1, 1,000 ppb of the internal standard 1-AG-d_5 and butylated hydroxytoluene as an antioxidant agent in a final concentration of 50 µg/mL.

6.3. Vortex the samples for 1 min and then sonicate them in an ultrasonic water bath for 15 min on ice.

6.4. Vortex the samples again twice for 1 min and centrifuge them for 10 min at 2,000 *x g* to induce the phase separation.

6.5. Collect the lower phase and collect it in a clean tube, dry it under nitrogen and re-suspend the solid residue in 100 µL of ACN.

7. Endocannabinoid analysis by HPLC-MS/MS

7.1. Use liquid chromatography coupled with an ESI triple quadrupole mass spectrometer to detect and quantify 2-AG from nematode samples.

7.2. Use the following conditions for the reversed-phase HPLC: from 0 to 0.5 min H_2O:ACN (40:60), from 0.5 to 6.5 min from H_2O:ACN (40:60) to (25:75), from 6.5 to 7.5 min H_2O:ACN (25:75), from 7.5 to 8.0 min from H_2O:ACN (25:75) to (40:60); from 8.0 to 12 min H_2O:ACN (40:60).

7.3. Maintain the column temperature at 40 °C and set the autosampler tray temperature at 10 °C.

7.4. Set these ionization conditions: positive-ion mode; drying gas (N_2) temperature: 300 °C; drying gas flow rate: 10 L/min; nebulizer pressure: 10 UA; Cap. Voltage, 4 kV.

7.5. For the analyte detection use MRM with the following transitions: m/z 379.2→289.2 for 2-AG and m/z 384.2→289.2 for 1-AG-d_5.

8. Endocannabinoid quantification in worms

8.1. Use deuterated internal standard 1-AG-d_5 and calculate the peak-area ratios of the analyte to the internal standard.

8.2. Use the transitions 384.2 m/z → 287.2 m/z for 2-AG and 379.2 m/z → 287.2 m/z for 1-AG-d_5.

8.3. Calculate the concentration of the endogenous 2-AG by comparing to the peak-area ratios of the deuterated standard using the concentration value of the standard.

REPRESENTATIVE RESULTS:

An isotopically labeled analog was successfully synthesized from commercially available d_8-glycerol and arachidonic acid using a 3-step synthetic method (**Figure 2 and 3**). These steps are straightforward not requiring sophisticated equipment, specially controlled conditions or expensive reagents. Thus, this method is robust and could be successfully extended to synthesize monoacylglycerides containing different fatty acids.

1-AG-d_5 was structurally characterized using nuclear magnetic spectroscopy. ^{1}H NMR showed the characteristic multiplet at 5.44-4.93 ppm which integrates for the eight vinyl protons of the arachidonoyl chain and the triplet at 2.40 ppm, corresponding to the two protons of the alpha position to the carbonyl group. In ^{2}D NMR it is also possible to see a 2.9-2.7 ppm multiplet assignable to the five deuterium of the glycerol portion.

The chemically synthesized 1-AG-d_5 was used as an internal standard in *C. elegans* samples. The standard was added to the samples before extraction, and then extracted with the endogenous lipids using a straightforward method adapted from Folch.[24] This modified method provides a high recovery value of the standard as showed by HPLC quantification.

The method was optimized using the transitions 384.2 m/z → 287.2 m/z for 2-AG and 379.2 m/z → 287.2 m/z for 1-AG-d_5 where the glycerol molecules are lost **(Figure 4)**.

The limits of detection (LOD) and quantification (LOQ) were calculated for the standard using a calibration curve resulting in values of 5 ppb and 16.6 ppb respectively. The retention time for the standard was 6.8 minutes.

2-AG endogenous from the *C. elegans* samples was successfully detected and quantified by isotopic dilution with the chemically synthesized 1-AG-d_5 using high-performance liquid chromatography coupled to mass spectrometry (HPLC MS/MS) **(Figure 5)**.

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Since the original concentration of the deuterated standard in sample 1 and 3 was of 1000 ppb each, from the peak-area ratio it is possible to calculate the endogenous concentration of 2-AG in the sample of 340 ppb for sample 1 and 360 ppm for sample 3, giving an average of 350 ppm **(Figure 6)**.

FIGURE AND TABLE LEGENDS:

Figure 1: Summary of the complete method: synthesis, worm sampling and quantification. To

achieve a successful quantification of the endogenous 2-AG it was necessary to synthesize its deuterated analog using a three-step sequence. Afterwards it was added to worm samples, extracted and analyzed by HPLC-MS/MS. Used as an internal standard, the synthetic of 1-AG-d_5 was the tool to quantify the endogenous metabolite.

Figure 2: Synthetic Scheme for the obtainment of 1-AG-d_5. 10 mg of the deuterated analog were obtained using a three steps method involving protection of the glycerol-d_8, acylation with arachidonic acid and deprotection.

Figure 3: Chemical structure of the isotopically labeled 2-AG analog.

Figure 4: Selected fragmentations for quantification of 1-AG-d_5 and 2-AG.

Figure 5: HPLC chromatograms for 1-AG-d_5 and 1-AG as pure standards and as internal standards in a worm sample. It is possible to analyze retention times and see that the worm appears not to have endogenous 1-AG and that it would only have 2-AG but the standard 1-AG-d_5 would still work as a good analytical standard for quantification by isotopic dilution. The transitions used were 384.2 m/z → 287.2 m/z for 2-AG and 379.2 m/z → 287.2 m/z for 1-AG-d_5.

Figure 6: Peak-area ratios for the deuterated standard and the endogenous 2-AG. The ratios were calculated as a quotient between the peak areas of 2-AG and 1-AG-d_5 respectively for two isolated samples, both with the deuterated standard added previous to extraction.

DISCUSSION:

Endocannabinoids are a class of lipids that have been implicated in the regulation of dauer formation in *C. elegans*.[7] More specifically, the synthesis of polyunsaturated fatty acids (PUFAs) is important for cholesterol trafficking and for the reproductive development of worms. It was also revealed that 2-arachidonoylglycerol (2-AG), an arachidonic acid containing endocannabinoid, is responsible for restituting the dauer larva to its normal cycle in worms that have an impaired cholesterol metabolism.[7]

Given the recently discovered importance of 2-AG in the enhancement of cholesterol trafficking and other biological processes and the scarce information of how lipids influence this process, it was necessary a reliable detection method for this endocannabinoid. The successful development of a simple and robust synthetic method, reported here, to obtain the deuterated analog 1-AG-d_5 was a key step of this protocol.

Most of the reported methods to quantify monoacylglycerols involve the use of commercially available analytical standards which are usually expensive and unstable under regular storage conditions making them inconvenient for research groups who need bigger quantities of standards and to always have a fresh stock of them. They are also unreachable for lower budget laboratories. However, this method sorts this obstacle by proposing a synthesis of the standard using more accessible starting materials.

It is also remarkable that opposite to other reported methods which use deuterated analytical standards of 2-substitued monoacylglycerols that suffer acyl-migration under many conditions and as a consequence, two chromatographic peaks are seen that may affect the relative quantification by isotopic dilution,[25] our method efficiently uses a 1-substitued deuterated analytical standard which is a single isomer and does not undergo acyl-migration.

The synthetic method is straightforward without sophisticated conditions needed making it ideal to be performed in any laboratory with minimal equipment and access to reactants, even in a reduced budget. It is also a simple technique that can be use by any scientist working in the field, without the need of an organic synthesis special training.

The worm sample preparation is the conventional one without further complications and the lipid extraction method to obtain the final samples is a modification from the Folch´s protocol[24] that allows for the better recovery values since it does not require chromatographic column purification.

The critical step is to make sure that the sample preparation and lipid extraction are performed adequately to have a good recovery of the standard that is detectable. It is also important to make fresh stock solutions monthly to assure the conditions of the standard and to check by NMR-spectroscopy or LC-MS that the standard is still pure and has not undergone oxidation or degradation.

The only limitation of this technique relies in the expansion to other studies that might have endogenous 2-AG concentrations lower than the presented LOQ. In that case, the method should be modified to make sure that the concentration falls between the limits.

In the case of a failure during the protocol where no visible chromatographic signal of the standard or the recovery value of the standard after the extraction is lower than expected, it is recommended to repeat the whole process of sample preparation and lipid extraction.

In summary, this new procedure describes a straightforward and reproducible way of detecting and quantifying 2-AG that will help to answer some of the unanswered questions regarding the role of this endocannabinoid in *C. elegans.*

Since the synthetic route involves the synthesis of a protected deuterated glycerol building block that is finally acylated with the arachidonic acid in the last step, this method could be expanded to the synthesis of deuterated standards of other monoacylglycerols, diacylglicerols, phospholipids and structurally related metabolites.

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DISCLOSURES:

The authors declare that they have no conflict of interest.

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