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Optimized extraction of 2-arachidonyl glycerol and anandamide from aortic tissue and plasma for quantification by LC-MS/MS

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Abstract

Atherosclerosis is a disease characterized by plaque formation due to an accumulation of fat, cholesterol, and immune cells in the walls of arteries. If a plaque ruptures, an occlusive thrombosis may form that causes either a heart attack or stroke. Macrophages express CB-2 receptors, and are one type of immune cell that plays a role in plaque destabilization and rupture. Endocannabinoids anandamide (AEA) and 2-arachidonyl glycerol (2-AG) have been found to have activity on CB-1 and CB-2 receptors throughout the body and immune system. In this study, we investigated several sample preparation options for the LC-MS quantification of AEA and 2-AG from plasma and aortic tissue. The extractions considered included liquid–liquid (LLE), solid-phase (SPE), and supported liquid (SLE). Some extraction protocols yielded high analyte recovery and prevention of 1-AG/2-AG isomerization. Our results indicate that a liquid-liquid extraction using toluene yields the highest recovery for both analytes, coupled with low ionization suppression in the mass spectrometer. This extraction and corresponding LC-MS/MS assay provides a simple, high throughput mechanism for the quantification of 2-AG and AEA in matrices relevant to the study of endocannabinoids' role in atherosclerosis.

Keywords

Anandamide; AEA; 2-Arachidonoylglycerol; 2-AG; Endocannabinoids; Liquid Chromatography; Mass Spectrometry; LC-MS; Toluene Extraction

1 Introduction

Anandamide (AEA) and 2-arachidonyl glycerol (2-AG) are endogenous ligands of cannabinoid receptors (CB) [1]. Cannabinoid type 1 (CB-1) receptors have been found in

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Practical applications

We developed an extraction method for AEA and 2-AG from plasma and aortic tissue samples and an LC-MS/MS assay for quantification of these compounds to help understand the role of endocannabinoids and CB-2 receptors in atherosclerosis. This assay can be applied toward the measurement of endocannabinoids, AEA and 2AG, in aortic tissue and plasma.

The authors have declared no conflict of interest.

higher concentrations in the central nervous system [2], while cannabinoid type 2 (CB-2) receptors have been found to be primarily located in the periphery and immune system [3]. CB-2 receptors have been found to modulate pain initiation [4] and provide anti-inflammatory effects [5]; it is the anti-inflammatory action of CB-2 receptors that is thought to provide beneficial effects in atherosclerosis. Monocyte-derived macrophages have been associated with maladaptive, non-resolving inflammatory response that leads to plaque formation with additional cells, lipid, and matrix. Some atherosclerotic lesions undergo necrotic disruption, triggering acute thrombotic vascular disease, including ischemic heart disease, myocardial infarction, cerebrovascular disease, and sudden cardiac death [6]. Activation of CB-2 receptors has been found to inhibit macrophage inflammatory activity [7], theoretically providing useful effects on the initial development, disease progression, or advanced stage plaque rupture of atherosclerosis.

Available literature contains several methods for extraction of endocannabinoids from various biological matrices. AEA was first extracted from porcine brain [8]. Subsequent studies extracted these compounds for examination in plasma [9, 10], serum [11], female reproductive tissues [12], saliva [12], breast milk [12], and spinal cord tissue [13]. Additionally, these compounds are of interest in the study of lipidomic profiles and the role of endocannabinoids in cell signaling [14]. Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) protocols dominate published methods. The chemistry of these analytes is highly suited for SPE extraction using hydrophilic-lipophilic balanced (HLB) cartridges [10] and C18 cartridges [15]. Published LLE protocols exploit solvent mixtures or moderate polarity solvents such as dichloromethane/isopropyl alcohol, chloroform/methanol, chloroform/water, and ethyl acetate [9, 10, 16, 17]. Finally, a study examining AEA and 2-AG in patients with endotoxic shock found that these compounds readily adsorb to polymyxin-B (PMB) beads, and utilized this property to create a unique solid-liquid combination phase extraction [11].

The isomerization of 2-AG to the biologically inactive 1-arachidonyl glycerol (1-AG) presents a significant analytical challenge to the development of an extraction method for 2-AG [18, 19]. This isomerization is characterized by acyl migration and is thought to be highly correlated with samples containing serum albumin, high pH values, or elevated temperatures [20]. Additionally, compound hydrolysis can lead to poor recovery and analytical precision. The two enzymes associated with AEA and 2-AG degradation in vivo are fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) [21, 22]. A reliable extraction would provide protection against hydrolysis and minimize 2-AG isomerization. In this study, we present a toluene-based LLE method for simultaneous 2-AG and AEA coupled with targeted LC-MS/MS-based quantification. Experiments were done using plasma and aortic tissue isolated from low density lipoprotein receptor (Ldlr) deficient mice (a murine model of atherosclerosis) with low endogenous levels of AEA and 2-AG [23]. A recent review of analytical methods related to the quantification of AEA in 2-AG in biological matrices did not identify work published for extraction of these compounds from aortic tissue [24].

2 Materials and methods

2.1 Chemicals and materials

All deuterium-labeled internal standards were obtained from Cayman Chemical (Ann Arbor, MI, USA). The labeled standard for 2-arachidonyl glycerol- d_8 was declared to have chemical purity of 95% as a 9:1 mixture of 2-AG and 1-AG. The labeled standard for arachidonyl ethanolamide- d_8 was declared to have chemical purity of 95%. The deuterium incorporation for both analytes was declared at 99% and 1% for d_1 – d_8 and d_0 , respectively. All labeled standards were supplied in a solution of ethyl acetate (AEA) and acetonitrile (2-AG). LC-MS grade distilled water, acetone, acetonitrile, methanol, 0.1% formic acid in acetonitrile was from Honeywell Burdick & Jackson (Muskegon, MI, USA). 0.1 M MES buffered saline was obtained from Bioworld (Dublin, OH, USA). Borosilicate glass tubes were obtained from VWR International (West Chester, PA, USA). 0.22 μ m Costar Spin-X nylon filter centrifuge tubes were obtained from Corning, Inc. (Tewksbury, MA, USA). Affi-Prep Polymixin-B Matrix was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Oasis HLB (1 cc, 30 mg) extraction cartridges were obtained from Waters Corporation (Milford, MA, USA). C18 SPE cartridges (1 cc, 30 mg) were obtained from Supelco (St. Louis, MO, USA). 15 mL polypropylene conical tubes were obtained from BD Biosciences (Bedford, MA, USA). Isolute SLE+ 1 mL columns were obtained from Biotage (Charlotte, NC, USA). HPLC grade toluene, methyl-*t*-butyl ether, dichloromethane, and isopropyl alcohol were obtained from Thermo Fisher Scientific (Waltham, MA, USA). PMSF (phenylmethanesulfonylfluoride) was obtained from Amresco (Solon, OH, USA).

2.2 Instrumentation and conditions

The Shimadzu liquid chromatography system consisted of two LC-20AD pumps with UFLC-XR upgrade, SIL-20AHT autosampler, CTO-20A column oven, DGU-20A₃ degasser, and CBM-20A Communications module. This system was coupled to the Shimadzu IT-TOF mass spectrometer with an electrospray (ESI) source (Columbia, MD). Instrumental control and spectral processing were accomplished using LCMS solution, version 3 from Shimadzu. Separation was performed using a Phenomenex Kinetex C18 column (2.6 μ m, 2.1 $\times$ 100 mm²) (Torrance, CA). The column oven was maintained at a temperature of 40°C. The HPLC was set up with a gradient elution as follows: 0–1 min 75% B, 1–4 min ramp up to 100% B, 4–7 min re-equilibrate to 75% B. Mobile phase A was 0.2% acetic acid in water and 0.1% formic acid in acetonitrile was used for mobile phase B. Injection volume was 10 μ L of the reconstituted sample, with a flow rate 0.200 mL/min. Detection was achieved with a direct MS/MS method in positive electrospray (+ESI) mode with a detector voltage maintained at 1.70 kV. Samples were maintained at a temperature of 4°C in the autosampler tray prior to injection. Channels employed for detection were: AEA- d_8 (m/z 356.24), AEA (m/z 348.29), d_8 -2-AG (m/z 386.28), and 2-AG (m/z 379.23). Each channel had an optimized ion accumulation time based on the sensitivity of that analyte. The nebulizing gas was nitrogen (N_2) flowing at 1.5 mL/min, while argon was used as the collision gas. The rough pump area vacuum was maintained at 75–85 Pa, IT area vacuum at 1.2×10^{-2} Pa, and TOF area vacuum at 1.4×10^{-4} Pa. The interface, heat block, and curved-desolvation line (CDL) were maintained at a temperature of 200°C. Dwell time was set at 250 ms for AEA and 2-AG and 50 ms for their octadeurated equivalents.

2.3 Preparation of spiked standards

Plasma samples were prepared by adding all standards and octadeuterated internal standards to plasma to reach a final volume of 500 μ L. Tissue samples were prepared by obtaining the weight (milligrams) of the aortic tissue and adding an appropriate amount milliliter of MES buffered saline for complete homogenization, then 500 μ L aliquots of homogenate were removed for spiking with internal standards. Biological samples were collected from control male mice (>8-wk old; Jackson Labs, Bar Harbor, ME, USA). Aortas were dissected and cleared of adventitial tissue, snap frozen in liquid nitrogen, and stored at -80°C until analysis. Mice were housed in the Animal Research Facility at East Tennessee State University in a pathogen-free, humidity-controlled, and temperature-controlled room. They were maintained on a standard chow diet (Ralston Purine, St. Louis, MO, USA) with water provided ad libitum. All animal procedures were approved by and conducted in accordance with the guidelines administered by the Institutional Animal Care and Usage Committee of East Tennessee State University and in conformity with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals. Tissue homogenization was performed in 15 mL polypropylene tubes using the Fisher Scientific Tissuemiser for 5 min. AEA and 2-AG concentrates were diluted using LC-MS grade methanol into stock solutions to be added to plasma and tissue blanks for extraction and analysis. Standards of each compound were diluted to obtain final stock solution concentrations of 100 and 10 $\mu\text{g/mL}$. Desired calibration curve concentration points were 10, 7.5, 5, 2.5, 1, and 0.5 $\mu\text{g/mL}$. All calibration samples were spiked with volumes of AEA- d^8 and 2-AG- d^8 standards to yield final concentrations of 5 and 10 $\mu\text{g/mL}$, respectively.

2.4 Extraction method development

Several extraction protocols were tested on spiked plasma samples and evaluated for AEA and 2-AG recovery versus a 50 $\mu\text{g/mL}$ standard in acetonitrile. The extraction methods with the highest nominal recovery of each analyte from plasma were then applied to extraction of AEA and 2-AG from aortic tissue homogenate. The procedures for each extraction tested are summarized in Table 1. Each protocol shared a common final step: evaporation to dryness under nitrogen and reconstitution in a small volume (100 μL) of acetonitrile. Final reconstituted samples were filtered using 0.22 μm nylon filter microcentrifuge tubes.

2.5 Evaluation of matrix effects

One milliliter of stock solution (100 $\mu\text{g/mL}$) was prepared as noted above. This solution was infused into the mass spec source, while blank aortic tissue extracts prepared using the LLE-toluene protocol were injected using the autosampler [25]. The stock solution infusion was accomplished using a Harvard Apparatus Pump 11+(Holliston, MA) running at a 0.010 mL/min flow rate, and a 100 μL Hamilton syringe (Reno, NV), and a solvent mixing tee at the mass spec source.

2.6 Statistics

Data from animal studies were analyzed by Student's t -test or ANOVA followed by the Bonferroni test for comparisons between groups, as appropriate. All data are presented as

means $\pm$ SD. $P < 0.05$ values were considered statistically significant. All data analyses were performed using Sigma-Plot software (Systat Software, Inc., San Jose, CA).

3 Results and discussion

Pre-treatment protein precipitation steps using 1 mM PMSF in acetone and 1% acetic acid in acetonitrile (both at freezer temperatures) were investigated with several of the extraction protocols. We found that acetonitrile-based protein precipitations led to higher levels of isomerization of 2-AG to 1-AG, compared to acetone-based precipitation. This could be an interaction with the acetonitrile, as previously reported [⁹], or could be related to the presence of PMSF in the acetone [¹¹]. The aforementioned isomerization is chromatographically demonstrated in Fig. 1.

The protocols executed using the Isolute SLE+ cartridges were adapted from the User Guide provided by Biotage (ISOLUTE™ SLE+ User Guide, www.biotage.com). While some conditions of SLE+ showed promising results, there was not a buffer/elution solvent combination that was advantageous for both AEA and 2-AG (Fig. 2). For each analyte, a buffer step involving 0.1% formic acid in water was preferred, but AEA performed better using the DCM/IPA elution buffer while more 2-AG was recovered via elution with MTBE. It is important to note that these protocols did not involve the use of the protease inhibitor, PMSF.

SPE using C18 cartridges yielded very poor results. AEA was not detectable, and 2-AG was barely discernable from the background noise. Additionally, samples extracted using the PMB suspension beads yielded sub-optimal results. While the PMB protocol yielded excellent recovery (>90%) for 2-AG with no indication of isomerization, AEA was not recovered. AEA could have been lost during one of the bead washing steps, or could have been strongly retained to the PMB beads. Given the time-consuming nature of the PMB suspension extraction, despite its novelty, it was not further optimized.

Of the LLE solvents tested, toluene yielded the highest recoveries for both AEA and 2-AG (>80%) for each. A similar recovery was noted for the SPE-Oasis HLB extraction from plasma; thus, these two protocols were tested for their applicability to preparation of aortic tissue samples spiked with AEA and 2-AG. Recovery was compared at two concentration levels (10 and 50 $\mu\text{g/mL}$). Additionally, calibration curves using the octadeuterated internal standards were prepared in duplicate in the range of 50–0.5 $\mu\text{g/mL}$ ($R^2 > 0.98$) for extraction via LLE-toluene and SPE-HLB. Recovery of 2-AG was acceptable from aortic tissue for both LLE-toluene and SPE-HLB extractions. Using LLE-toluene, 89% of 10 $\mu\text{g/mL}$ 2-AG and 88% of 50 $\mu\text{g/mL}$ 2-AG were recovered. Likewise, using SPE-HLB, 86% of 10 $\mu\text{g/mL}$ 2-AG and 81% of 50 $\mu\text{g/mL}$ 2-AG were recovered. The lower recovery using solid-phase extraction was found to be due to breakthrough loss of 2-AG during the wash steps, even at lower concentrations. Recovery for AEA was very good using the LLE-toluene extraction, with 93% of 10 $\mu\text{g/mL}$ and 89% of 50 $\mu\text{g/mL}$ AEA detected versus a spiked standard of the same concentrations. The recovery at the higher concentrations could have been increased by increasing the volume of the extraction solvent. The recovery of AEA using the SPE-HLB method exceeded 100% (114% at the 10 $\mu\text{g/mL}$ level and 109% at the 50 $\mu\text{g/mL}$ level).

The explanation for this can be found in the examination of the chromatography under both of these extraction conditions (Fig. 3). The peak shape for AEA when the aortic tissue sample is prepared using HLB-SPE has discernable fronting, which could contribute to a falsely high recovery value. The peak shapes for both analytes are acceptable following the LLE-toluene extraction. Finally, the limit of quantification (LOQ), based on a signal-to-noise (s/n) ratio of 10:1 for the LLE extraction was 0.5 µg/mL for AEA and 1 µg/mL for 2-AG from aortic tissue homogenates; however, the LOQ was much higher for 2-AG (5 µg/mL) using the HLB SPE tubes. LOQ from the plasma matrix was 0.5 µg/mL for both analytes using the toluene extraction. Further verification of ionization suppression effects associated with the aortic tissue matrix extracted using toluene LLE indicates a region of suppression between 0.2 and 1.6 min, which does not interfere with the elution of AEA (RT =2.4 min) and 2-AG (RT =3.2 min) (see Fig. 4). This acceptable ionization suppression profile for toluene is likely linked to its limited tendency to extract phospholipids from biological matrices [9].

To evaluate the optimized extraction and LC-MS/MS methods in a biologically relevant tissue, AEA and 2-AG levels in aortas of low density lipoprotein receptor (Ldlr^{-/-}) null mice fed a high fat diet (HFD) or normal chow for 12 wk were quantified (Table 2). Diluted versions of some samples had to be re-analyzed for AEA concentrations in order to fall within the calibration range. Concentrations of AEA and 2-AG were initially calculated in µg/mL homogenate, and corrected for addition of MES buffer during homogenization to result in µg analyte/mg tissue. Statistically significant enhancement of both AEA (~1.5-fold) and 2-AG (1.9-fold) levels in aortas of Ldlr^{-/-} mice fed a HFD for 12 wk compared those fed normal chow was detected in these samples.

4 Conclusions

Plasma was used as a template matrix for the comparison of several extraction protocols geared toward recovery of AEA and 2-AG for LC-MS/MS analysis. Promising protocols were then applied to extraction of AEA and 2-AG from spiked aortic tissue, given that this is a biologically relevant matrix suitable for the study of these endocannabinoids as biomarkers for atherosclerosis. Our results indicate that a simple liquid-liquid extraction using toluene is suitable to achieve >85% recovery of these analytes from spiked aortic tissue homogenate, with no indication of 2-AG to 1-AG isomerization. Additionally, the LC-MS/MS method utilized in this study provides high sample throughput (4 min chromatographic separation) and sufficient clearance from the ionization suppression region to help ensure data reproducibility. This assay could realistically be applied to the study of AEA and 2-AG concentrations in murine plasma and aortic tissue.

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Abbreviations

1-AG	1-arachidonyl glycerol
2-AG	2-arachidonyl glycerol
AEA	arachidonyl ethanolamide/anandamide
C18	octadecyl
CB	cannabinoid receptor
DCM	dichloromethane
EA	ethyl acetate
ESI	electrospray ionization
FAAH	fatty acid amide hydrolase
HLB	hydrophilic-lipophilic balance
IT-TOF	ion trap-time of flight
IPA	isopropyl alcohol/2-propanol
LC	liquid chromatography
LLE	liquid–liquid extraction
MAGL	monoacylglycerol lipase
MES	2-(N-morpholino) ethane-sulfonic acid
MS	mass spectrometry
MTBE	methyl- <i>t</i> -butyl ether
PMB	polymixin-B bead
PMSF	phenylmethanesulfonylfluoride
RT	retention time
SLE	supported liquid extraction
SPE	solid phase extraction

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Figure 1.

Mass chromatogram of (a) 1-AG/2-AG isomerization produced in plasma extract following pre-treatment with acetonitrile-based protein precipitation versus (b) non-isomerized 2-AG found in plasma extract following pre-treatment with acetone-based protein precipitation.

Figure 2.

Extraction recovery from various SLE+ protocols, calculated as a ion count percentage compared to a 10 µg/mL standard of AEA and 2-AG ($n=3$ for each condition, shown as average $\pm$ standard deviation).

Figure 3. Mass chromatograms of AEA and 2-AG extracted from aortic tissues following (a) LLE with toluene and (b) SPE using Waters Oasis HLB cartridges (10 $\mu\text{g/mL}$ concentration of each).

Figure 4. Ionization suppression region associated with homogenized aortic tissue matrix for AEA (m/z 348.29; upper trace) and 2-AG (m/z 379.23; lower trace) mass spectrometric channels and following LLE with toluene.

Table 1

A summary of method steps employed for various extraction tests of AEA and 2-AG from spiked plasma

Extraction type	Sample pre-treatment	Extraction steps
Isolute SLE+ (Biotage)	Samples mixed in 1:1 ratio of plasma/0.1% aqueous formic acid or plasma/water for pH adjustment	Pre-treated samples were applied to SLE+ cartridges in a 12-position vacuum manifold and allowed to sit for 5 min. Four different elution solvents were explored, including methyl- <i>t</i> -butyl ether (MTBE), dichloromethane (DCM), dichloromethane/isopropanol mixture (DCM/IPA; 95:5), and ethyl acetate (EA). Vacuum was applied for 2 min to collect eluate.
C18 SPE (Supelco)	Samples mixed in a 1:2 ratio of plasma/PMSF saturated saline	SPE columns were set up in a 12-position vacuum manifold. The columns were conditioned with methanol (2.5 mL) and ion-free water (1 mL). Samples were then loaded and allowed to flow under gravity for 15 min. The columns were washed 55% methanol (1.5 mL) and ion-free water (1 mL). Elution was carried out with acetonitrile.
Oasis HLB SPE (Waters)	Samples mixed in a 1:2 plasma/protein precipitation solution, vortex-mixed, and centrifuged at 10 000 rpm for 5 min. Two protein precipitation solutions were considered, including 1mM PMSF in acetone and 1% acetic acid in acetonitrile.	SPE columns were set up in a 12-position vacuum manifold. Supernatants from protein precipitation were collected and transferred to extraction cartridges and allowed to flow under gravity for 15 min. The columns were washed with 40% methanol (1 mL) and ion-free water (1 mL). Two elution solvents were explored, including DCM/IPA 95:5 and acetonitrile.
Liquid-liquid extractions	Samples mixed in a 1:2 plasma/protein precipitation solution, vortex-mixed, and centrifuged at 10 000 rpm for 5 min. Two protein precipitation solutions were considered, including 1mM PMSF in acetone and 1% acetic acid in acetonitrile.	Supernatants from protein precipitation were collected and transferred to 2 mL microcentrifuge tubes. One milliliter aliquots of extraction solvents were added to supernatants and vortex mixed for 30 s. Extraction solvents considered included chloroform, chloroform/methanol (2:1), and toluene. Following phase separation, the organic layer was transferred to a clean borosilicate glass tube.
Polymixin-B (PMB) bead suspension extraction	Samples mixed in a 1:2 plasma/PMSF saturated saline in 15 mL polypropylene tubes	500 µL of polymixin-B bead (PMB) suspension was added to the plasma/saline mixture and allowed to shake on a Fisher tube shaker table for 1 h while being maintained at 4°C (to prevent analyte degradation). Samples were washed with two 2 mL aliquots of saline and centrifuged at 10 000 rpm for 5 min. Elution from the beads was carried out with two rinses with absolute ethanol (200 µL aliquots).

Table 2

Aortic endocannabinoids in Ldlr-null mice

Endocannabinoid	Chow	HFD
AEA	152.6 ± 28.76	228.1 ± 3.32 *
2-AG	0.449 ± 0.227	0.865 ± 0.144 **

Values are means expressed as µg/mg wet tissue weight ± SD. *n*=4 for each group.

* *P*<0.05 versus chow diet.

** *P*<0.001 versus chow diet.