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Simultaneous determination of 2-arachidonoylglycerol, 1-arachidonoylglycerol and arachidonic acid in mouse brain tissue using liquid chromatography/tandem mass spectrometry

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Endocannabinoids (ECs), such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG), modulate a number of physiological processes, including pain, appetite and emotional state. Levels of ECs are tightly controlled by enzymatic biosynthesis and degradation *in vivo*. However, there is limited knowledge about the enzymes that terminate signaling of the major brain EC, 2-AG. Identification and quantification of 2-AG, 1-AG and arachidonic acid (AA) is important for studying the enzymatic hydrolysis of 2-AG. We have developed a sensitive and specific quantification method for simultaneous determination of 2-AG, 1-AG and AA from mouse brain and adipose tissues by liquid chromatography/tandem mass spectrometry (LC/MS/MS) using a simple brain sample preparation method. The separations were carried out based on reversed phase chromatography. Optimization of electrospray ionization conditions established the limits of detection (S/N=3) at 50, 25 and 65 fmol for 2-AG, 1-AG and AA, respectively. The methods were selective, precise (%R.S.D. <10%) and sensitive over a range of 0.02–20, 0.01–10 and 0.05–50 ng/mg tissue for 2-AG, 1-AG and AA, respectively. The quantification method was validated with consideration of the matrix effects and the mass spectrometry (MS) responses of the analytes and the deuterium labeled internal standard (IS). The developed methods were applied to study the hydrolysis of 2-AG from mouse brain extracts containing membrane bound monoacylglycerol lipase (MAGL), and to measure the basal levels of 2-AG, 1-AG and AA in mouse brain and adipose tissues.

Keywords: 2-arachidonoylglycerol; 2-arachidonoylglycerol hydrolysis; quantification; liquid chromatography; tandem mass spectrometry; brain sample preparation

Introduction

Endocannabinoids (ECs), such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG), modulate a number of physiological and pathological processes, including pain, schizophrenia, stroke, obesity, Alzheimer's disease, multiple sclerosis, cancer, appetite, neural development, immune cell activation, retrograde neural signaling and emotional state.[1–11] 2-AG acts as a full agonist for both CB1 and CB2 cannabinoid receptors.[12–16] In contrast, AEA acts as a partial agonist at the CB receptors.[15–17] The endogenous levels of 2-AG present in rat brain were found at more than 170- to 1000-fold higher than AEA.[17,18] The extent of EC accumulation in tissue and corresponding receptor activation is tightly controlled by enzymatic biosynthesis and degradation *in vivo*. The hydrolysis of AEA leading to the production of arachidonic acid (AA) and ethanolamine is primarily carried out by a single enzyme fatty acid amide hydrolase (FAAH) and is well characterized.[19,20] Multiple enzymes, including FAAH,[21] neuropathy target esterase (NTE),[22] and monoacylglycerol lipase (MAGL),[23] have been shown to degrade 2-AG *in vitro* to AA and glycerol. However, there is limited knowledge about the enzymes that terminate signaling of the major brain EC, 2-AG, in the brain, although MAGL has generally been assumed to be the main contributor in this process. Using a functional proteomic approach, Blankman *et al*.[24] showed that ~85% of brain 2-AG hydrolase activity can be attributed to MAGL, and the remaining 15% is mostly catalyzed by ABHD6 and ABHD12. Accumulated evidence suggests that inhibition of specific EC hydrolyzing enzymes might be useful as a promising therapeutic approach to relieve discrete symptoms without producing side effects.[25] Functional studies involving the physiological roles of ECs have been mainly focused on AEA and 2-AG.[18,26,27] Development of reliable extraction and quantification methods to accurately measure the levels of ECs from biological tissue samples has been a growing interest in many areas of neuroscience research to facilitate studies of the signally mechanism and to evaluate the potential inhibitors of specific EC biosynthesizing or hydrolyzing enzyme.

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The extraction of 2-AG from brain tissue reported in the literature was primarily based on solvent-based extraction, which requires further time consuming sample preparation procedures, including chemical derivatization for gas chromatography-mass spectrometry (GC/MS),[7,28,29] and solid phase extraction (SPE) for liquid chromatography-mass spectrometry (LC/MS or LC/MS/MS).[5,30–32] Selected ion monitoring (SIM) mode was performed in the GC/MS or LC/MS methods to measure 2-AG, identified based on a derivative fragment ion or on the molecular ion. Kingsley *et al*. were the first to describe the use of a LC/MS/MS method, based on the high specificity of multiple reaction monitoring (MRM) mode, for analyzing 2-AG via a silver adduct.[31] Many reported methods in the literature for quantification of 2-AG did not include validation data. Richardson *et al*. described validation of a LC/MS/MS method for the simultaneous measurement of AEA, 2-AG and other related compounds.[32] However, the ion suppression and the MS response of the endogenous analytes and their internal standards (ISs) in biological matrixes were not discussed. In addition, the reported measurements of 2-AG were based on the combined concentrations of 2-AG and 1-AG, in which they were not chromatographically separated.

An analytical technique was previously reported for simultaneous measurement of 2-AG, 1-AG and AA, based on the use of high-performance liquid chromatography (HPLC) coupled with ultraviolet (UV) detection.[33] The detection of these analytes relies only on their chromatographic retention times (RTs), without method validation.

Coupling MS with chromatographic separation techniques provides the advantage of detecting these analytes by both their chromatographic RTs and the corresponding molecular ions. The MRM scan mode on a triple quadrupole mass spectrometer monitors specific product ions from dissociation of their parent molecular ions. This approach, combined with the specific LC RT of analytes, provides a sensitive and selective analysis, which is unique for individual analytes.

Here we report the development and validation of a simple brain and adipose tissue sample preparation method in conjunction with a sensitive and selective quantitative method for simultaneous determination of 2-AG, 1-AG and AA from mouse brain tissues using LC/MS/MS. This rapid and cost-effective sample preparation method consisted of a simple protein precipitation step by using acetonitrile (ACN). The HPLC separation was carried out using reversed phase chromatography. The developed and validated methods were applied to study the hydrolysis of 2-AG from mouse brain extracts containing membrane-bound MAGL, and to measure the basal levels of 2-AG, 1-AG and AA in mouse brain and adipose tissues.

Experimental

Reagents and chemicals

2-AG, 1-AG, AA and 2-AG-d8 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Formic acid, LC grade water, methanol (MEOH) and ACN were obtained from EM Science (Gibbstown, NJ, USA). Reagents used to prepare the buffer solution used for the *in vitro* activity assay were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid was from J. T. Baker (Phillipsburg, NJ, USA). Mouse MAGL cDNA was purchased from Open Biosystems (Huntsville, AL, USA).

Preparation of membrane extracts from mouse brain

The brain tissue was harvested from male C57BL/6J mice (Charles River Laboratories International, Inc., Wilmington, MA, USA), and immediately kept frozen in dry ice. Frozen tissues were stored at −80 °C. For membrane-bound preparation of MAGL, a Dounce homogenization was performed in Tris buffer (50 mM Tris-HCl, pH 7.5), containing protease inhibitors and 150 mM NaCl. Following brief sonication, a low-speed centrifugation was performed at 4 °C to remove cell debris. The supernatant was centrifuged at high speed for 40 min at 4 °C in order to isolate membrane-bound MAGL. This high-speed centrifugation was repeated twice. The final pellet was re-suspended in Tris buffer, sonicated and kept at −80 °C until use.

Enzymatic activity assays

2-AG (50 μM) was incubated in a buffer consisting of 54 mM HCl, 1.1 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 0.5% bovine serum albumin (BSA) (pH 7.4) using either recombinant MAGL (rMAGL) protein (expressed and purified from *Escherichia coli*) (unpublished) or membrane-bound MAGL (5 μg membrane protein) as discussed above. At time points of 0, 10, 30, 60, 90 and 120 min, 100 μl of sample was removed from the incubation and 200 μl of ACN (pH 3.0) was added to stop the enzymatic reaction. The pH of the sample was adjusted to 3.0 using formic acid to stabilize 2-AG against a possible post-incubation chemical acyl migration reaction to 1-AG. Samples were centrifuged. Twenty micro-litter of 2-AG-d8 (IS) solution was added into an aliquot of 80 μl of supernatants. The samples were kept at −80 °C until LC/MS/MS analysis. The degradation (both enzymatic and chemical) of 2-AG was determined by simultaneously monitoring 2-AG, 1-AG and AA using LC/MS/MS.

Mouse brain and adipose tissue sample preparation

Male C57BL/6J mice (Charles River Laboratories International, Inc., Wilmington, MA, USA) between 8 and 12 weeks old and weighing 20–30 g were housed in groups of 4 at a temperature of 20 °C +/− 5 °C and were kept on a 12 : 12-h light : dark cycle with free access to food and water for at least 1 week prior to testing. Animal protocols were performed in accordance with NIH guideline for the Care and Use of Laboratory Animals and approved by Wyeth's IACUC.

Mouse brain and adipose samples (~100 mg) were homogenized in 100 μl of 0.02% trifluoroacetic acid (TFA, pH 3.0) (1000 mg tissue/ml). The brain homogenate, 20 μl of IS and 4 ml of ACN were mixed in a silanized glass tube. The mixture was centrifuged (3000 rpm, 4 °C) for 15 min. The supernatant was transferred into a clean silanized glass tube and evaporated to dryness under nitrogen using a Zymark TurboVap evaporator at 35 °C. The residue was reconstituted into 100 μl of ACN and vortexed, followed by a brief centrifugation to remove any precipitates. The samples were transferred into HPLC vials for storage at −80 °C before analysis by LC/MS/MS.

To determine the extraction efficiency of 2-AG, 1-AG, AA and 2-AG-d8 from brain homogenate, analyte-spiked brain samples and analyte-spiked control brain samples were prepared using the sample preparation procedure discussed above. The analyte-spiked brain samples were prepared by spiking homogenate from 100 mg brain tissue with 20 μl of each stock solution of 2-AG, 1-AG, AA and 2-AG-d8, followed by adding 4 ml of ACN. The samples

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were vortexed, and then centrifuged. The supernatant was dried down under N_2 and reconstituted into 100 µl of ACN. The analyte-spiked control brain samples were prepared by aliquoting 100 mg of brain tissue, followed by homogenization, protein precipitation by ACN and centrifugation. The supernatant was dried under N_2 and reconstituted into 20 µl of ACN, plus 20 µl of each stock solution of 2-AG, 1-AG, AA and 2-AG-d8 to make a final volume of 100 µl.

Preparation of standard and quality control samples

Serials stock solutions of 2-AG, 1-AG and AA were prepared by dilution of 1 mg/ml of ACN solutions of each with ACN, as 2-AG is known to be more stable in ACN.[32] A stock solution of 2-AG-d8, used as IS, was prepared at a concentration of 20 µg/ml in ACN. The stock solutions were stored at −80 °C. Standard and quality control (QC) samples were prepared by spiking 20 µl of each corresponding stock solution of 2-AG, 1-AG and AA and 20 µl of IS into 100 µl of water. The spiked solution and 4 ml of ACN were mixed in a silanized glass tube and transferred into a clean silanized glass tube and evaporated to dryness under nitrogen at 35 °C. The residuals were reconstituted into 100 µl of ACN. Seven-point calibration curves were constructed for 2-AG, 1-AG and AA in the concentration range of 0.02–20, 0.01–10 and 0.05–50 ng/mg tissue for 2-AG, 1-AG and AA, respectively.

Validation was carried out by performing calibration experiments over three non-consecutive days. For each day, calibration samples were prepared in duplicate at seven concentration levels according to the procedure described above. QC samples at three different concentration levels were prepared separately in duplicate for method validation.

Liquid chromatography

An Agilent 1100 series HPLC system (Hewlett-Packard GmbH, Waldbronn, Germany) was used in this study. The system consists of two quaternary pumps, a vacuum degasser, a temperature controlled autosampler and a thermostated column compartment.

The chromatographic separation was carried out using a Chromolith RP-18E (100 × 3.0 mm i.d) (Merck KGaA, Darmstadt, Germany), maintained at 40 °C. The mobile phase consisted of solvent A: 0.2% acetic acid in water–methanol (H_2O : MEOH=95 : 5, v/v) and B: 0.2% acetic acid in water–methanol (H_2O : MEOH=5 : 95, v/v). The HPLC analysis started with 40% B for 0.5 min, then followed by a gradient from 40% to 95% B in 1 min and subsequently hold at 95% B for 6.5 min. The flow rate was 0.5 ml/min. The HPLC flow was split before the MS and ∼0.2 ml/min effluent was directed into the electrospray ionization (ESI) source of the mass spectrometer. The injection volume was 20 µl.

Mass spectrometry

On-line LC/MS/MS analyses were performed using a Micromass Quatro Micro tandem quadrupole mass spectrometer (Waters, Beverly, MA, USA) operated in positive ESI mode with the ion source temperature of 125 °C. The positive ESI ((+)ESI) conditions for 2-AG, 1-AG, AA and 2-AG-d8 were optimized to a desolvation temperature of 350 °C, a spray voltage of 3.5 kV and a cone voltage of 20 V. Nitrogen was used as both desolvation (1000 l/h) and nebulizer gas (fully open). The pressure of the argon collision gas was set at 5 psi and adjusted to an analyzer pressure of 2.0–3.0 × 10^{-4} mbar. MRM modes were used for analysis. The MRM analyses were preformed by passing molecular ions through the first quadrupole (Q1) followed by collisional dissociating the molecular ions in the second quadrupole (collision cell – Q2). A selected product ion, based on intensity and structure characteristics, was isolated by the third quadrupole (Q3) and detected with the photomultiplier set at 650. The MRM transitions of m/z 379 → 287 for 2-AG and 1-AG, 305 → 93 for AA and 387 → 295 for 2-AG-d8 were simultaneously monitored. This approach provides a sensitive and selective analysis that is unique for non-isomeric analytes. The concentrations of 2-AG, 1-AG and AA were determined by calculating their corresponding peak area ratio to that of the IS using a linear fit weighting to the calibration curve.

Results and Discussion

Optimization of brain sample preparation method

Current literature suggests that conversion of 2-AG to 1-AG occurs through a chemical acyl migration reaction under particular experimental conditions.[33,34] Various solvent systems, such as chloroform, chloroform/methanol and ethyl acetate/hexane, were used in the literature for extraction of 2-AG from brain homogenates.[31,32,34–37] The stability of 2-AG during brain and adipose tissue preparation using these solvents including ACN were investigated. Our studies showed that more than ∼40% of 2-AG was converted to 1-AG when chloroform was used as the extraction solvent (data are not shown). The acyl migration converting 2-AG to 1-AG (or 2-AG-d8 to 1-AG-d8) was minimized when ACN or ethyl acetate/hexane was used. Figure 1 shows a comparison of the MRM traces for 2-AG-d8, 1-AG-d8 and AA-d8 from the mouse brain tissue extracts spiked with 2-AG-d8 (Fig. 1(a) and (c)), and from the ACN solutions of 2-AG-d8 (Fig. 1(b)) or AA-d8 standards (Fig. 1(d)). The brain extracts were prepared by spiking with 2-AG-d8 immediately after the brain tissues were homogenized, acidified and protein precipitated with ACN. As shown in Fig. 1(d), AA-d8 eluted at the RT of 6.79 min from the ACN solution of AA-d8, but it was not observed from the brain extracts spiked with 2-AG-d8 ((Fig. 1(b)). 1-AG-d8, eluted at the RT of 6.23 min, was originally observed in the ACN solution of 2-AG-d8 (Fig. 1(b)) [a fresh solution from Cayman Chemical (Ann Arbor, MI, USA). The percent ratios of [2-AG-d8] : [1-AG-d8] was 94±0.46 : 6±0.4 (n = 5) in both the brain extracts spiked with 2-AG-d8 (Fig. 1(a)) and the ACN solution of 2-AG-d8 (Fig. 1(b)). These observations showed that the enzymatic degradation of 2-AG-d8 to AA-d8 or the acyl migration converting 2-AG-d8 to 1-AG-d8 did not occur during the brain sample preparation process.

Extraction of 2-AG from brain tissues using ethyl acetate/hexane displayed high extraction recovery for 2-AG that required additional purification steps using SPE[31,32] to remove some extracted proteins or lipids, which plugged the column during the analysis. However, SPE sample preparation methods are complex and time consuming, which may limit the overall throughput. A modified approach described in this report using 40-fold excess of cold ACN compared to the sample volumes to extract 2-AG from mouse brain tissue homogenates provided optimal recovery of 2-AG. Moreover, ACN effectively removed the proteins and lipids in the brain and adipose tissue homogenates without the need for further sample clean up.

There has been a technical challenge to study the recovery of ECs from a biological matrix owing their endogenous nature. Richardson *et al*. reported two approaches to determine the recoveries of ECs with the consideration of the endogenous

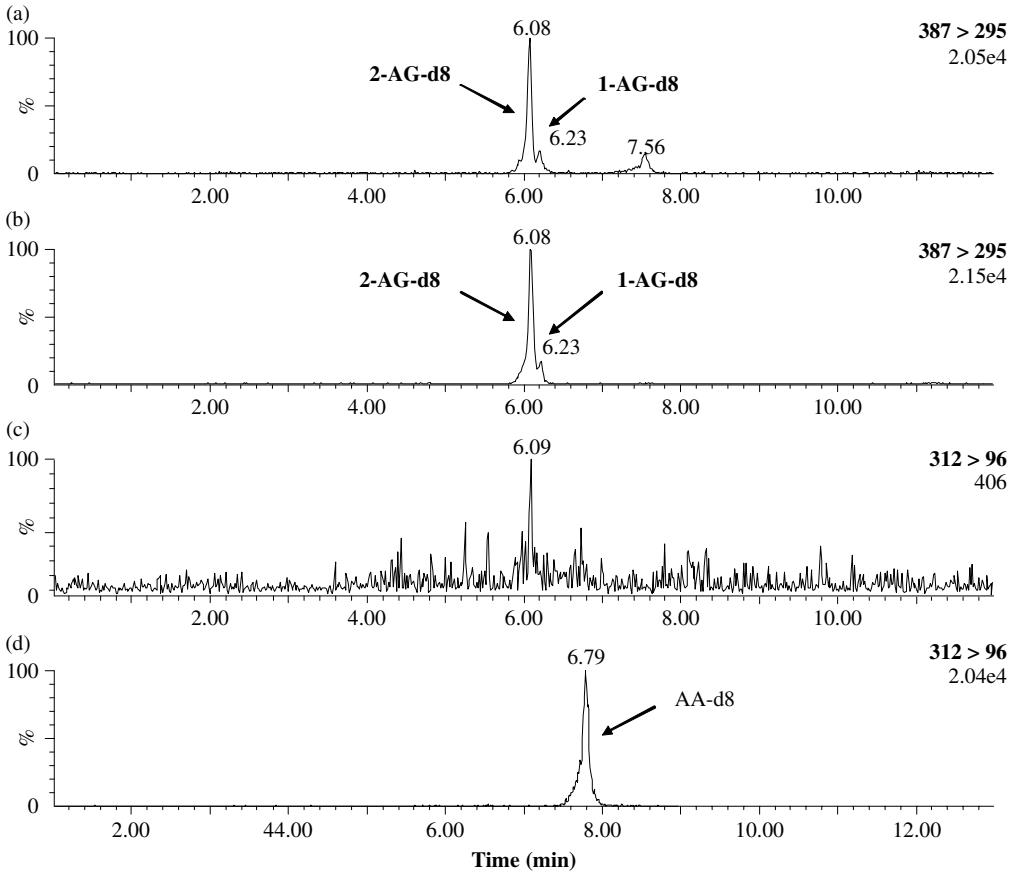

Figure 1. The MRM ion chromatograms of: a) 2-AG-d8 and 1-AG-d8 from the mouse brain tissue extracts spiked with 2-AG-d8, (b) 2-AG-d8 and 1-AG-d8 from the ACN solution of 2-AG-d8, c) AA-d8 from the mouse brain tissue extracts spiked with 2-AG-d8 and d) AA-d8 from the ACN solution of AA-d8.

levels of ECs.[32] A modified approach was used in our study to consider both the endogenous levels of 2-AG, 1-AG and AA and the matrix effects during ESI ionization. In our approach, the recovery of analytes was evaluated by comparing the area ratios of an analyte peak in analyte-spiked brain samples to that in analyte-spiked control brain samples. These samples were prepared by using the same sample preparation procedure as described in the experimental section. However, for the analyte-spiked brain samples, 20 μl of corresponding stock solutions of each analyte and IS were spiked before adding ACN for sample preparation. For the analyte-spiked control brain samples, 20 μl of corresponding stock solutions of each analyte and IS were spiked in the reconstitution step after ACN protein precipitation. This approach minimized the difference of the matrix effect between the analyte-spiked brain samples and analyte-spiked control brain samples under (+) ESI ionization, which might cause false positive or negative results. The recoveries of spiked 2-AG, 1-AG and AA were evaluated after subtracting the basal levels of 2-AG, 1-AG and AA measured from the blank brain samples. Recoveries of >80% were achieved for 2-AG, 1-AG, AA and 2-AG-d8 in these studies (data are not shown). The developed method also provided high recovery of AEA from the brain tissue (unpublished).

Optimization of LC/MS/MS conditions

The HPLC method was optimized to ensure the separation of the analytes. It was particularly important with regard to separation of 2-AG and 1-AG, because they share the same molecular weight and the same MRM transition (379 → 287). 1-AG could be formed by an aryl migration reaction of 2-AG, which could occur under some particular experimental conditions. The combined measurement of 1-AG and 2-AG without HPLC separation was used in most quantification methods for 2-AG reported in the literature.[31,32,38,39] However, our study showed that the MS response of 1-AG under (+) ESI MRM was 2 times higher than that of 2-AG. The combined measurement of 1-AG and 2-AG in LC/MS/MS analysis could significantly affect the measurement accuracy of 2-AG, especially in enzymatic activity assays, in which the concentrations of 2-AG and 1-AG were comparable.

Mobile phase systems containing 28% phosphate buffer was used by Saario *et al*. to separate 2-AG and 1-AG.[33] However, this mobile phase system is not ideal for ESI, since it could significantly reduce the ionization efficiency due to ion suppression. A number of reversed phase methods were evaluated. We found that using a Chromolith RP-18E column in combination with a mobile phase system consisting of H_2O and MEOH with 0.2% acetic acid provided the optimal separation of 1-AG and 2-AG. It is a common knowledge that use of MEOH as mobile phase solvent generates higher column back pressure compared to ACN. The low back pressure on Chromolith columns compared to other reversed phase columns provided an advantage in our use of a methanol-containing solvent system. Furthermore, the ionization efficiency of 1-AG and 2-AG was 2 times higher when MEOH was used as a mobile phase solvent instead of ACN. As a result, our method provided the optimal HPLC conditions and the optimal MS responses.

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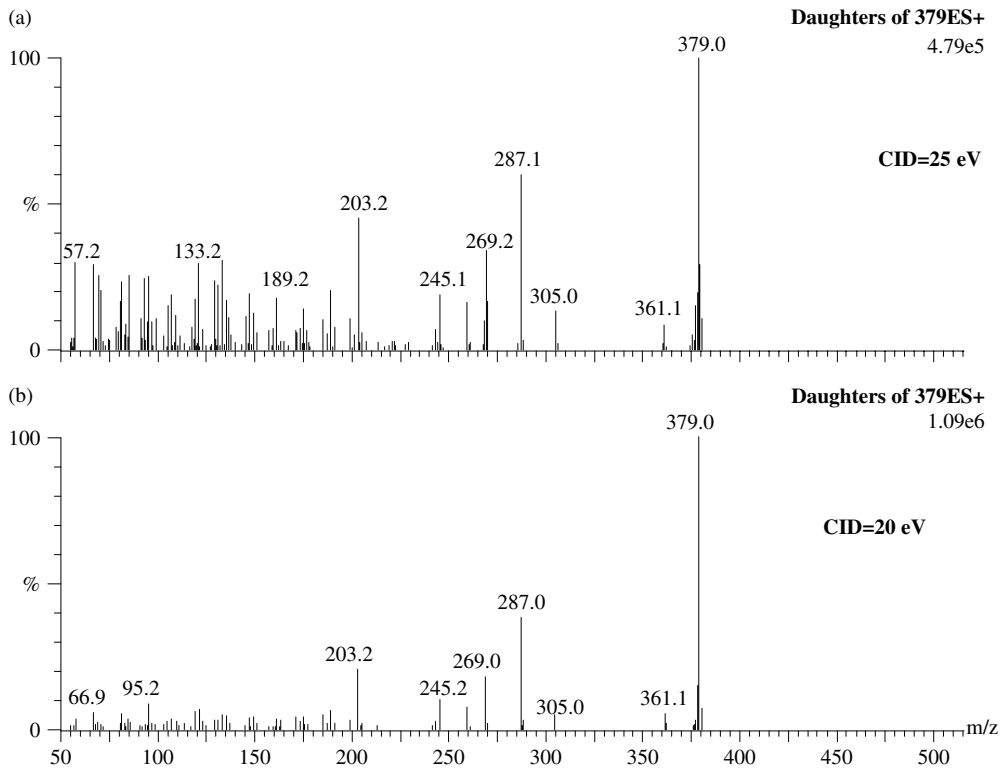
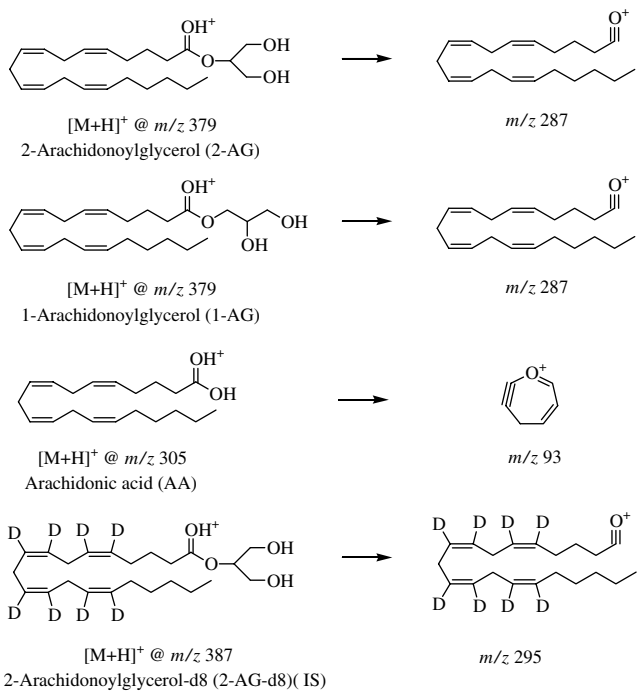


Figure 2. ESI product ion spectra of $[M+H]^+$ at m/z 379 for 2-AG using a collision energy of: a) 25 eV and b) 20 eV.

The ESI product ion spectra of the protonated molecular ion of 2-AG ($[M+H]^+$ at *m/z* 379) by using collision energies (CE) of 20 and 25 eV are shown in Fig. 2. Dissociation of the molecular ion of 2-AG produced a most abundant product ion at *m/z* 287 by using a CE of 20 eV. Numbers of low-mass fragment ions, which may not be structural specific product ions, were produced when the CE was increased to 25 eV, while the abundance of the ion at *m/z* 287 was decreased. The fragment ion at *m/z* 287 produced at a CE energy of 20 eV was the most abundance ion among all fragment ions observed in a CE energy range of 15–35 eV. In addition, the ion of *m/z* 287 was formed by a neutral loss of glycerol from the molecular ion, which was a structural characteristic ion for 2-AG (Scheme 1). Dissociation of the protonated molecular ions of 1-AG and 2-AG-d8 generated the fragment ions of *m/z* 287 and 295, respectively, by a neutral loss of glycerol from the corresponding molecular ions as well (Scheme 1). Dissociation of the protonated molecular ion of AA ($[M+H]^+$ @ *m/z* 305) produced mainly low-mass fragment ions (Fig. 3). The ion at *m/z* 93 was the most abundant fragment ion from the dissociation of the molecular ion of AA (Scheme 1). Therefore, the MRM transitions of *m/z* 379 → 287 for 2-AG and 1-AG, 305 → 93 for AA and 387 → 295 for 2-AG-d8 were chosen for quantitative analysis. 2-AG-d8 was used as the IS for quantification of all three analytes. AA-d8 was not selected as an IS for AA, since AA-d8 formed mainly $[M+Na]^+$ ions instead of $[M+H]^+$ ions in our system. These structurally specific MRM transitions associated with the specific HPLC retention times were used to confirm the identity of 2-AG, 1-AG, AA and 2-AG-d8 in the samples.



Scheme 1. Dissociation pathways of: a) 2-AG, b) 1-AG, c) AA and d) 2-AG −d8.

Sensitivity and linearity

The selected mobile phase systems allowed these analytes to be ionized with high ionization efficiency under (+) ESI. The MRM mode provided a sensitive and selective analysis that was unique for individual compounds. The limits of detection (LOD) were 50, 25 and 65 fmol (S : N = 3 : 1) for 2-AG, 1-AG and AA, respectively. The lowest quantification levels (LOQ) were 100, 50 and 130 fmol

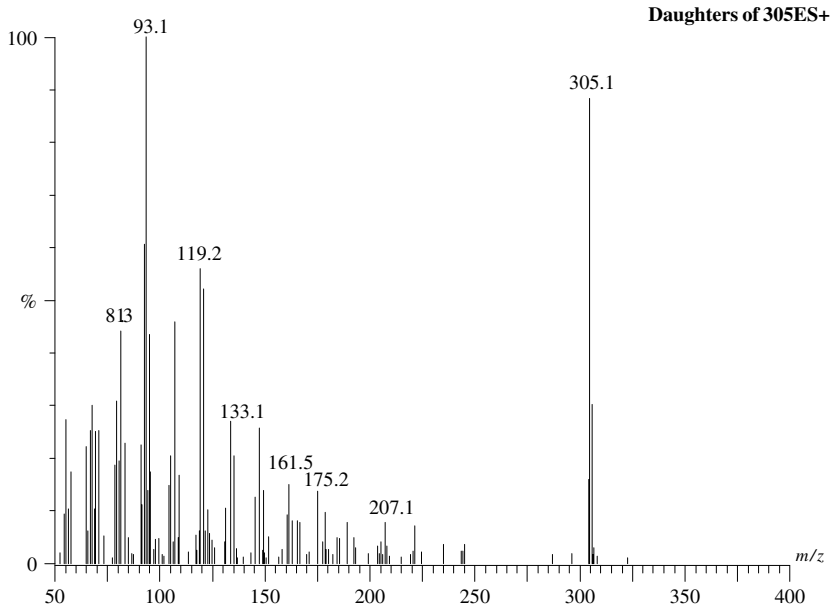


Figure 3. ESI product ion spectrum of $[M+H]^+$ at m/z 305 for AA (CE=30 eV).

Table 1. Intra-day and inter-day accuracy and precision of 2-AG, 1-AG and AA in QC samples

		Intra-day ($N = 6$)				Inter-day ($N = 18$)			
Analyte	Nominal Conc (ng/mg tissue) or (ng/μl)	Mean	Accuracy (%)	SD	%RSD	Mean	Accuracy (%)	SD	%R.S.D.
2-AG	0.1	0.10	104	0.01	6.1	0.10	104	0.01	5.4
	1	1.0	102	0.06	5.5	1.1	106	0.05	4.7
	10	10.2	102	0.4	3.7	10.2	102	0.4	3.8
1-AG	0.05	0.055	110	0.003	4.5	0.055	110	0.003	5.3
	0.5	0.51	102	0.02	4.3	0.50	99	0.02	4.6
	5	4.5	90	0.3	5.4	4.6	91	0.2	3.6
AA	0.2	0.19	95	0.02	10.6	0.20	99	0.02	8.5
	2	1.8	92	0.1	6.4	1.9	95	0.2	8.3
	20	19.4	97	0.8	7.7	18.9	95	0.6	6.6

(S : N = 10 : 1) for 2-AG, 1-AG and AA, respectively. The LOD (50 fmol) for 2-AG achieved in this method was more sensitive than the previously reported methods using GC/MS (1 pmol),[40,41] LC/MS (~5 pmol)[38] and LC/MS/MS (250 fmol).[32] The LOD at 13 fmol for 2-AG was reported by Kingsley *et al.* using silver adduct coordination tandem MS.[31] The LOD values for 1-AG and AA were not previously reported in the literature.

Seven-point standard curves were prepared to establish the calibration ranges for 2-AG, 1-AG and AA. The linear regression analysis with $1/x^2$ weighting was applied. Calibration curves of 2-AG, 1-AG and AA were linear in the concentration range of 0.02–20, 0.01–10 and 0.05–50 ng/mg tissue, respectively, with the correlation coefficients greater than 0.997. The quantification ranges were selected based on concentration ranges of these analytes present in the mouse brain studied here and also in our enzymatic activity studies.

Precision and accuracy

Precision and accuracy measurements were acquired for the QC samples at three different concentrations from three sets of samples, analyzed on three different days. The intra-day and in inter-day precision and accuracy are summarized in Table 1. The intra-day precision (%RSD) was better than 6.1%, 5.4% and 10.6% for 2-AG, 1-AG and AA, respectively. The value of the intra-day accuracy was in a range of 90–110%. The inter-day precision and accuracy were determined by pooling all validation data from all QC samples at each concentration. The inter-day precision was better than 5.4%, 5.3% and 8.5% for 2-AG, 1-AG and AA, respectively. The value of the inter-day accuracy was in a range of 91–110%. These results indicate good precision and accuracy.

MS response, matrix effect, selectivity and stability

Accurate quantification of endogenous chemicals has always been a technical challenge. The isotopic dilution methods using a deuterium labeled analog of an analyte have been traditionally employed for determination of ECs. However, there were few discussions regarding the MS response of the analyte and its deuterium labeled analog in the literature. Kingsley *et al.*[31] reported that the silver adducts of 2-AG and 2-AG-d8 showed a similar MS response at the same concentrations. Therefore, the

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Table 2. Matrix effects on ratios of 2-AG, 1-AG and AA to 2-AG-d8

Analyte	Nominal Conc of analyte (ng/mg tissue) or (ng/µl)	Area ratio [analyte]/[2-AG-d8] In brain	In solvent	*Accuracy (%)
2-AG	1	0.89	0.81	110
	10	7.99	8.39	95
	20	14.7	16.0	92
1-AG	0.1	0.18	0.17	106
	1	2.53	2.64	96
	10	31.4	33.7	93
AA	0.5	0.33	0.30	110
	2.5	1.67	1.72	97
	25	22.1	19.8	111

* Accuracy = $[\text{ratio}]_{\text{brain}}/[\text{ratio}]_{\text{solvent}}$

response of 2-AG silver adduct and 2-AG-d8 silver adduct was taken as equal for quantification calculations using the isotopic dilution method.[31] In our study, the response ratio of 2-AG to 2-AG-d8 was determined by using 2-AG : 2-AG-d8 standard solutions with molar ratios of 1 : 10, 1 : 1 and 10 : 1. The 2-AG : 2-AG-d8 area ratios were 0.75 ± 0.015, 6.53 ± 0.131 and 64.4 ± 2.72, respectively ($n = 3$, mean±SD). Despite the structural similarity of 2-AG and 2-AG-d8, the MS response of 2-AG was found to be about sevenfold higher than 2-AG-d8 using (+) ESI MRM scan mode. This large difference in the MS response between the analyte and its deuterium labeled analog could significantly affect the measurement accuracy using the traditional isotopic dilution methods.[42]

In the present study, a different approach of using calibration standards prepared in pure solvents following the sample preparation procedure described in the experimental section and using 2-AG-d8 as IS was evaluated. In the study, both MS responses and matrix effects were evaluated by comparing the peak area ratios of each analyte to 2-AG-d8 in the standard solutions to that in the analyte-spiked brain samples at three different spiked analyte concentration levels. The basal levels of 2-AG, 1-AG and AA were measured using the aliquots of blank brain samples. It should be noted that due to the high basal level of 2-AG in brain tissue, the lowest spiked level of 2-AG at 1 ng/mg tissue was selected. The peak area ratios of spiked 2-AG, 1-AG and AA to 2-AG-d8 in brain samples were calculated after subtracting the basal levels of 2-AG, 1-AG and AA. As presented in Table 2, the peak area ratios of 2-AG, 1-AG and AA to 2-AG-d8 in ACN solutions and in brain samples were very similar. The accuracy determined by the percent ratios of [analyte] : [IS] in brain samples to that in ACN solutions were in the range of 91–111%. These observations indicated that even though there was a large difference between the MS responses of 2-AG and 2-AG-d8, the matrix effects on 2-AG and 2-AG-d8 in the same matrix were very similar. Our data suggest that use of calibration curves prepared in pure solvents and use of 2-AG-d8 as the IS provided reliable quantification for 2-AG, 1-AG and AA in brain tissue samples.

The MRM ion chromatograms of 2-AG (100 fmol), 1-AG (50 fmol), AA (130 fmol) and 2-AG-d8 (IS, 200 pmol) in a standard solution are presented in Fig. 4. 1-AG-d8 was observed with a relative amount of ~6% of 2-AG-d8 in the solution. Utilization of structurally specific MRM transitions associated with the specific HPLC retention times were used to confirm the identity of these analytes and IS.

The stabilities of 2-AG, 1-AG and AA spiked in control brain homogenates were evaluated at both 4 and 25 °C for 24 h. Our observations showed that all analytes were stable under these experimental conditions.

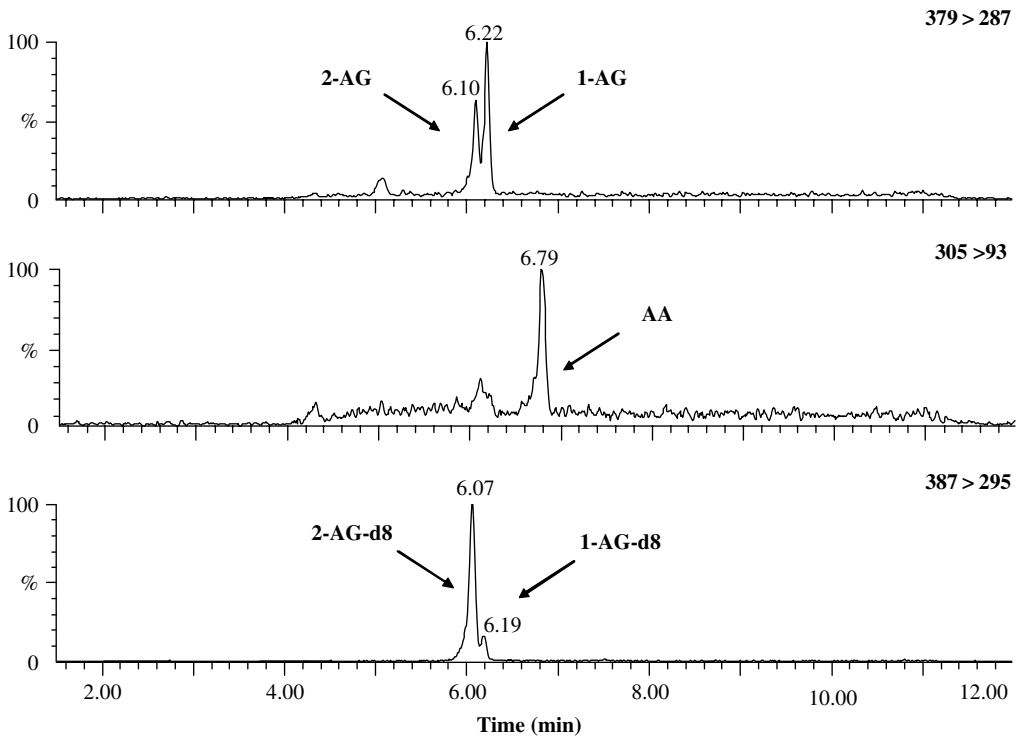
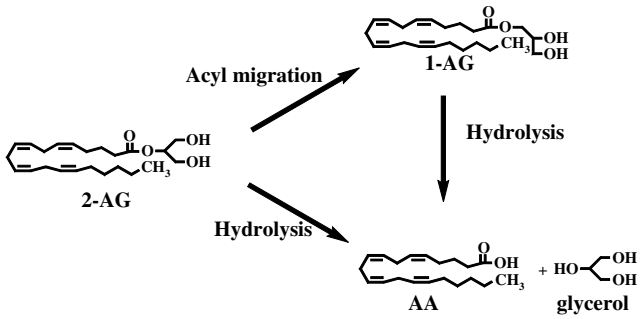


Figure 4. The MRM ion chromatograms of 2-AG (100 fmol), 1-AG (50 fmol), AA (130 fmol) and 2-AG-d8 (200 pmol) in an ACN solution.



Scheme 2. Hydrolysis and acyl migration reactions of 2-AG in mouse brain extracts containing membrane-bound MAGL.

Table 3. Acryl migration and hydrolysis of 2-AG (mean +SE) in buffer, mouse brain extract and purified rMAGL at 30-min incubation

	% 2-AG, 1-AG and AA to total species		
Compound	In buffer	In mouse brain extract	In purified rMAGL
2-AG	76±6.8	26±6.3	8.2±2.1
1-AG	24±3.0	5.9±0.6	6.5±0.4
AA	Not detected	68±7.2	85±4.5

Application

There is limited knowledge about the enzymes that terminate signaling of the major brain EC, 2-AG, in the brain, although MAGL has generally been assumed to be the main contributor in this process. The presented methods have been applied to understand EC signaling through their production and enzymatic degradation. The developed methods have been applied to study 2-AG hydrolysis *in vitro* and was also used to measure basal levels of 2-AG in mouse brain and adipose tissues.

Enzymatic activity for hydrolysis of 2-AG in mouse cerebellar membranes

As presented in Scheme 2, 2-AG could be enzymatically degraded to AA and glycerol by a hydrolysis reaction, where 1-AG, formed by acyl migration of 2-AG, could also be degraded to AA.[33] Saario *et al*. previously reported the investigation of monoglyceride lipase-like enzymatic activity for hydrolysis of 2-AG using an HPLC method.[33] In their method, the relative concentrations of 2-AG, 1-AG and AA were estimated on the basis of corresponding UV peak areas. In the present work, the degradation of 2-AG was investigated by incubating 2-AG in membrane-free buffer, rMAGL protein or membrane-bound MAGL prepared from the whole brain following the published procedure.[15] 2-AG, 1-AG and AA were monitored simultaneously using our validated LC/MS/MS method, which provides higher accuracy and sensitivity than the HPLC/UV method.

As illustrated in Table 3, 24% of 2-AG was converted to 1-AG after a 30-min incubation in membrane-free buffer, suggesting the formation of 1-AG by a chemical acyl migration reaction. AA was not observed when 2-AG was incubated with buffer alone, indicating that the formation of AA was enzyme dependent. These observations agreed with Saario's finding.[33]

The degradation of 2-AG was presented as the relative percent concentrations (μM) of each analyte compared to the total three

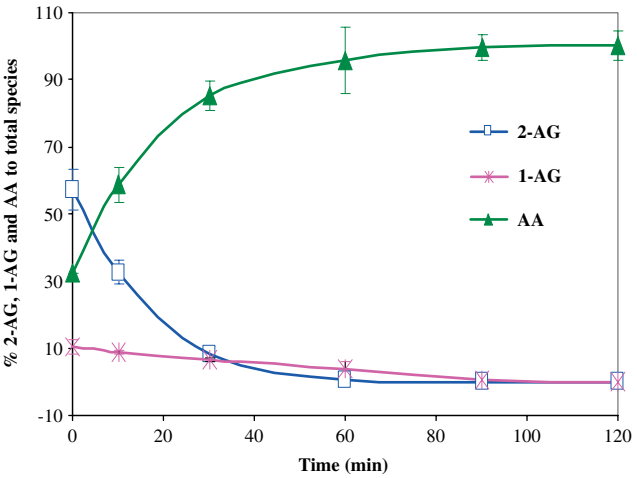


Figure 5. Acyl migration and hydrolysis of 2-AG in purified rMAGL.

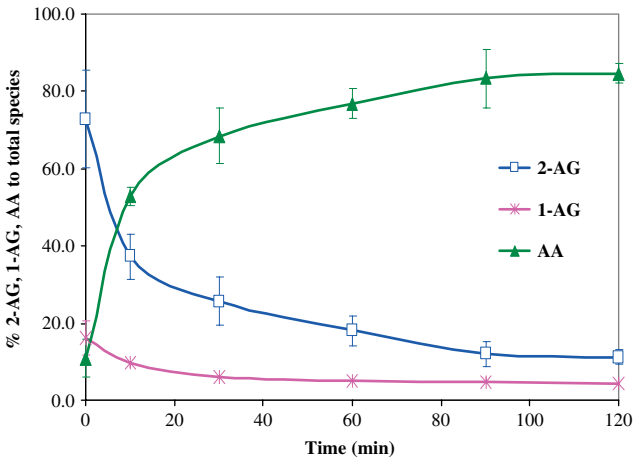


Figure 6. Acyl migration and hydrolysis of 2-AG in membranes of mouse brain extracts.

species of 2-AG, 1-AG and AA. The time course of degradation of 2-AG when incubated either with rMAGL protein or membrane-bound MAGL, prepared from the whole mouse brain, are shown in Figs 5 and 6, respectively. The relative concentration of 2-AG was 26 ± 6.3% and 8.2 ± 2.1% after a 30-min incubation with brain extract and rMAGL, respectively (Table 3). The relative concentration of 1-AG was 5.9 ± 0.6% and 6.5 ± 0.4% after a 30-min incubation with brain extract and rMAGL, respectively. The relative concentration of AA, formed by the hydrolysis of 2-AG and 1-AG, was 68 ± 7.2% and 85 ± 4.5% by brain extract and rMAGL, respectively. These observations suggested that the majority of 2-AG was hydrolyzed by MAGL. In comparison, Saario *et al*. reported the relative concentration of AA was ~20% after a 30-min incubation with rat cerebellar membranes.[33]

The basal levels of 2-AG, 1-AG and AA from mouse brain and adipose tissues

The sample preparation and LC-MS/MS methods reported here were applied to measure the basal levels of 2-AG, 1-AG and AA from mouse brain tissues. Due to the complex nature of the brain samples, two MRM transitions for each analyte and the IS were monitored simultaneously for the analysis of the brain extracts.

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Table 4. Comparison of literature reports of basal levels of 2-AG in mouse and rat extracts

Reference	Species/region	2-AG basal level (ng/mg)	2-AG basal level (pmol/mg)	Detection method
Bisogno *et al.* (1999)[29]	Different rat brain regions	1.5–5.3	4.0–14.0	GC/EI/MS
Fezza *et al.* (2002)[5]	Whole rat brain	1.7	4.5	LC/APCI/MS
Valenti *et al.* (2004)[30]	Different rat brain regions	5–15	13.2–39.7	LC/APCI/MS
Kingsley *et al.* (2003)[31]	Whole mouse brain	5.0	13.2	LC/ESI/MS/MS
Richardson *et al.* (2006)[32]	Different rat brain regions	4.1–11.3	10.8–29.9	LC/ESI/MS/MS
Current work	Whole mouse brain	3.6	9.5	LC/ESI/MS/MS

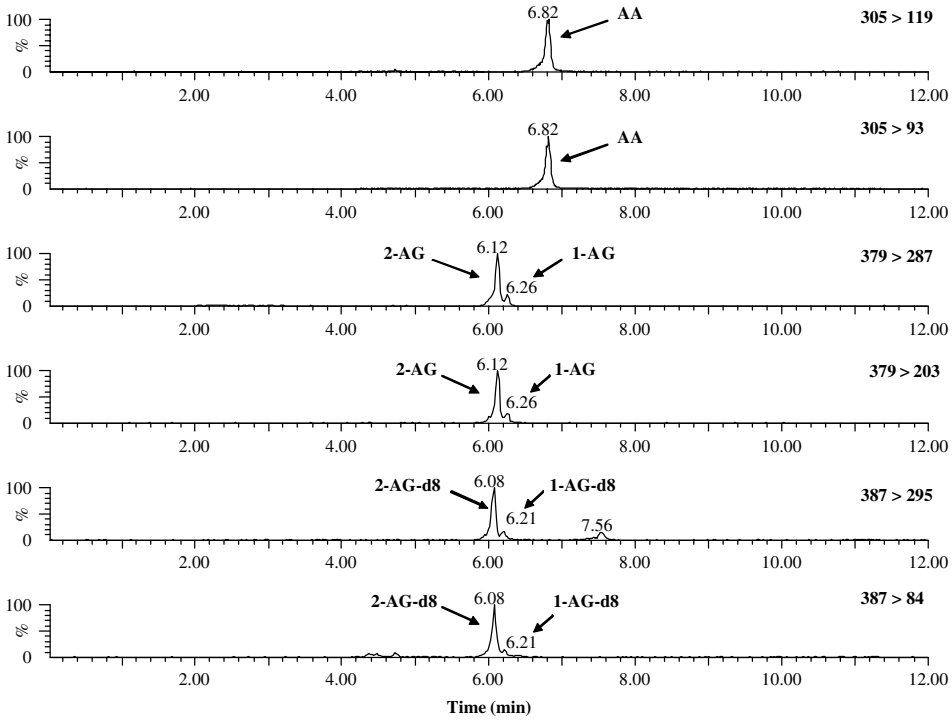

Figure 7. The MRM ion chromatograms of the basal 2-AG, 1-AG, AA and the spiked 2-AG-d8 (IS) from the mouse brain tissue extracts.

The MRM transitions were *m/z* 379 → 287 and 379 → 203 for 2-AG and 1-AG; *m/z* 305 → 93 and 305 → 119 for AA; and *m/z* 387 → 295 and 387 → 84 for 2-AG-d8. The identities of 2-AG, 1-AG, AA and 2-AG-d8 were confirmed by comparing their HPLC retention times and the MRM transitions with the standard compounds. The ion chromatograms of the 2-AG, 1-AG and AA from the mouse brain tissue extract and the spiked 2-AG-d8 are presented in Fig. 7. The basal levels of 2-AG, 1-AG and AA in mouse tissue were determined to be 3.6 ± 0.41, 0.34 ± 0.03 and 18.0 ± 2.1 ng/mg tissue, respectively. The basal levels of 2-AG, measured using GC/EI/MS, LC/APCI/MS and LC/ESI/MS/MS reported in the literature are summarized in Table 4. The basal level of 2-AG in the whole mouse brain described in this report is compatible with that reported by Kingsley *et al.* using the silver adduct LC/MS/MS method.[31]

The basal levels of 2-AG were also determined in mouse adipose tissue using our methods. We observed ten fold higher levels of 2-AG in the brain compared to the adipose tissue (Fig. 8) ($N = 3$, $P < 0.005$), which was consistent with the reported finding that MAGL activity was higher in adipose tissue compared to the brain tissue.

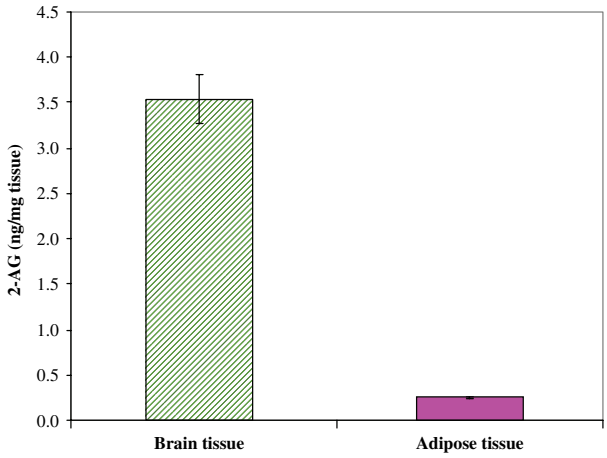

Figure 8. The basal levels of 2-AG in mouse brain and adipose tissue.

Conclusion

A brain sample preparation method and a reversed phase LC/MS/MS method have been developed for simultaneous

determination of 2-AG, 1-AG and AA in mouse brain and adipose tissue. The current method provides higher detection sensitivity than that reported using HPLC methods based on UV detection. In addition, the LC/MS/MS method provides higher accuracy by identifying the analytes using structurally specific MRM transitions associated with the selective HPLC retention times. The use of 40-fold excess of cold ACN for sample preparation provided optimal recovery of 2-AG, 1-AG and AA, and at the same time, effectively removed the proteins and lipids in the brain and adipose tissue homogenates. Chromatographic separation of 2-AG and 1-AG ensured the accurate quantification of 2-AG. The quantification method was validated with consideration of matrix effects on the MS responses of the analytes and IS.

These methods were used for both *in vitro* and *in vivo* to simultaneously determine the concentrations of 2-AG, 1-AG and AA. These methods appear to be sensitive and specific and have been successfully applied to measure 2-AG, 1-AG and AA concentrations in mouse brain and adipose tissue. Accurate measurement of 2-AG and its enzymatically catalyzed degradation products would be extremely useful in understanding the EC signaling and its regulation by enzymatic biosynthesis and degradation.

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