Quantitative LC-MS/MS method development and preliminary analysis of plasma endocannabinoid concentrations in humans

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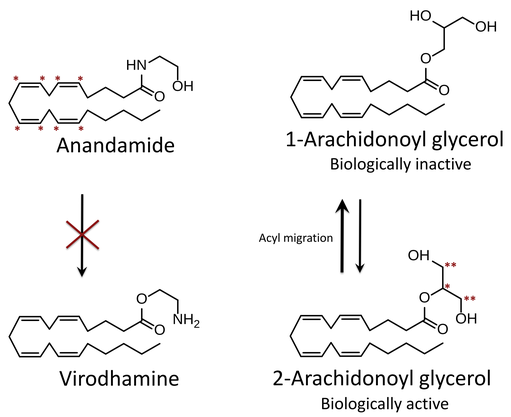
Introduction

Endocannabinoids (eCBs) are endogenous arachidonate-based lipid molecules released in the nervous system and may have a role in the pathophysiology of various brain disorders. Previous research has focused on anandamide (AEA) and 2-arachidonoylglycerol (2AG), as these are the two most prevalent eCBs involved in mood regulation, anxiety, mental state, and social functioning.

Some neuropsychiatric disorders, such as schizophrenia, major depression, and eating disorders, demonstrate atypical eCB activity indicating a possible role in multiple brain disorders, including neurodevelopmental conditions. In this study we focused on the development and refinement of a sensitive and robust LC-MS/MS based method to simultaneously and accurately monitor levels of AEA, 1AG and 2AG in human plasma samples.

Analytes



* Position of deuterium label on internal standard

SCHEME 1. Endocannabinoids. *2AG is known to undergo spontaneous isomerization to 1AG via acyl migration. The parallel transformation of AEA is rarely observed. Commercially available standards for AEA, 2AG, 1AG and their corresponding stable isotope labeled internal standards were purchased from Sigma-Aldrich, IsoSciences, C/D/N Isotopes Inc., and Cambridge Isotope Laboratories, Inc.*

Methods

Instrumentation

LC/MS: Accela 1250 LC, TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific)

Column: Acquity BEH C18 (Waters) (150 mm x 2.1 mm, 1.7 µm particle size)

Mobile phase A: 0.1% formic acid

Mobile phase B: 0.1% formic acid in ACN

Injection volume: 5 µl

Flow rate: 300 µl/min

Ionization mode: positive HESI

Scan mode: Selected Reaction Monitoring (SRM)

Data analysis: Xcalibur software (Thermo Fisher Scientific)

TABLE 2. LC gradient

Time [min]	%B
0	50
1	50
2	98
4	98
5	50
8	50

TABLE 3. Analytes. *Three major endocannabinoids and two corresponding deuterium-labeled standards were monitored, using 3 to 4 SRM transitions for each.*

Analyte	Abbreviation	MW [g/mol]	SRM Transitions
Anandamide	AEA	347.5	348.3 > 287.4, 203.4, 269.2, 91.0
***d8*-Anandamide**	AEA IS	355.6	356.3 > 294.3, 252.1, 206.1
2-Arachidonoyl glycerol	2AG	378.6	379.3 > 287.3, 269.3, 91. 0
***d5*-2-Arachidonoyl glycerol**	2AG IS	383.6	384.4 > 287.3, 269.3, 202.8
1-Arachidonoyl glycerol	1AG	378.6	379.3 > 287.3, 269.3, 91.0

LC-MS/MS

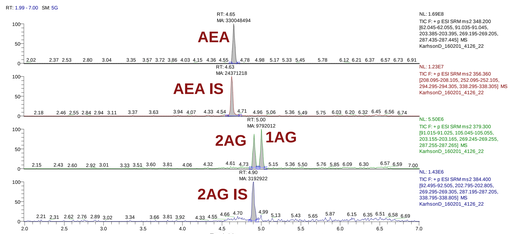


Figure 1. SRM chromatograms. *Baseline separation of isomeric arachidonoyl glycerols was necessary to enable monitoring of 2AG isomerization to 1AG.*

Calibration curve and LLOQ

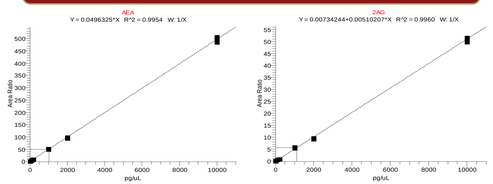


Figure 2. Calibration curve range: *0.1 pg/µl – 10,000 pg/µl prepared in plasma, equivalent to 0.5 pg to 50 ng on column*
Lower Limits of Quantitation (LLOQ) *in extracted plasma – defined as signal to noise ratio (S/N) of 10 to 1 – were 50 fg and 10 pg for AEA and 2AG, respectively.*

Sample preparation

Biological samples: Plasma samples from six participants ranging from 3 to 8 years of age were used for method development.

Two extraction procedures were tested:
SALLE – Salting-out assisted liquid-liquid extraction with acetonitrile and 5M ammonium formate
Toluene – Liquid-liquid extraction with toluene, drying under N_2

TABLE 2. Extraction comparison. *SALLE proved more reproducible than toluene extraction, and was applied to the analysis of >200 plasma samples from individuals with and without brain disorders.*

	SALLE	Toluene
Reagents	- 5 M AmF - 25 ng/mL AEA IS/2AG IS in ACN - 50 µg/mL standard stocks (AEA, 2AG) - QC levels: - QC L – 10 ng/mL - QC M – 200 ng/mL - QC H – 2500 ng/mL	- Toluene (HPLC garde) - 500 ng/mL AEA IS/2AG IS in ACN - 50 µg/mL standard stocks (AEA, 2AG) - QC levels: - QC L – 10 ng/mL - QC M – 200 ng/mL - QC H – 2500 ng/mL
Procedure	- Take 100 µl of sample - Add 200 µl of IS solution (ACN) - Add 50 µl of 5M AmF - Vortex for 1 min - Spin @ 13000 rpm for 5 min - Collect top organic layer and transfer to AS vial	- Take 50 µl of sample - Add 5 µl IS mix - Add 100 µl toluene - Vortex for 1 min - Spin @ 5000 rpm for 5 min - Collect top organic layer - Dry under N_2 - Reconstitute in 100 µl of 50%ACN/0.1% FA - Vortex for 1 min - Spin @ 13000 rpm for 5 min - Transfer to AS vial
Extraction yield – AEA/2AG	85%/90%	90%/95%
Matrix effect	AEA – not significant 2AG – signal enhancement	AEA - Signal suppression for QC L; signal enhancement for QC M and QC H 2AG - Signal enhancement - all QC levels
Sample prep – for 10 samples	20 min	45 min

Abbreviations: AmF - Ammonium formate , ACN - acetonitrile, FA - formic acid

Results

Isomerization

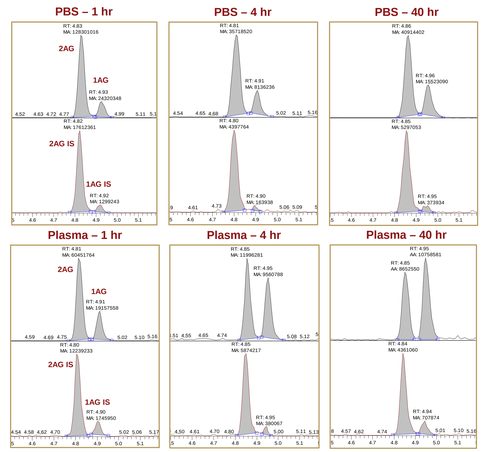


Figure 3. Isomerization. *Multiple injections of the same sample show isomerization progress over time. Extracted plasma components promote 2AG conversion to 1AG, while the process is slower in PBS. A peak for labeled 1AG was observed, even though it was not spiked into the samples. Both peak areas - 2AG IS and 1AG IS - were measured and the sum was used for IS response calculations.*

Conclusions

- In this study we established a sensitive LC-MS/MS method enabling efficient analysis of endogenous endocannabinoids in plasma, and evaluated two sample preparation procedures.
- Even though liquid-liquid extraction yield with toluene was slightly higher than with SALLE, SALLE was more reliable and reproducible across wide concentration ranges.
- Levels of AEA and 2AG detected in control plasma samples were within range of literature values.
- Isomerization of 2AG to 1AG was observed in all biological samples, regardless of extraction procedure used. The conversion rate was up to 60% in 40 hours. Levels of both 2AG and 1AG were measured.

References

1) Zoerner, A. et al. *J. of Chrom. B* **2012**, 161-171

Acknowledgments

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