**SUPPLEMENTARY MATERIAL**

**Determination of the Endocannabinoids Anandamide and 2-Arachidonoyl Glycerol with Gas Chromatography / Mass Spectrometry - Analytical and Pre-Analytical Challenges and Pitfalls**

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**Materials and Methods**

**Chemicals**

The following compounds were obtained from Cayman Chemical Company (Ann Arbor, MI, USA): arachidonoyl ethanolamide (AEA; 50 mg/mL in ethanol), arachidonoyl ethanolamide-D4 (AEA-D4; 1 mg/mL ethanolic solution), 2-arachidonoyl glycerol (2-AG; 10 mg/mL in acetonitrile (ACN)), 2-arachidonoyl glycerol-D5 (2-AG-D5; 1 mg/mL in ACN). *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA/TMCS), heptane and toluene were obtained from Sigma-Aldrich Chemie (Buchs, Switzerland), hexane and ACN from Merck (Darmstadt, Germany). All other solvents were of the best available grade.

**Collection and preparation of plasma samples**

A SOP developed and validated in our laboratory was strictly followed to collect blood samples and obtain plasma thereof. The volunteers, who were all staff members and co-workers of the Department of Clinical Research and the Institute of Clinical Pharmacology of the University of Bern, were instructed to refrain from eating and were only allowed to drink water in the 12 h preceding the sampling in order to ensure fasting blood samples. All samples were collected in the morning between 8 and 9.30 a.m. as follows: blood samples (2 x 9 mL) were collected into EDTA plasma tubes (Sarstedt, Sevelen, Switzerland) by venipuncture of the median cubital vein using a butterfly needle (Dispomed Witt, Gelnhausen, Germany). The plasma tubes were carefully mixed, placed on ice immediately and centrifuged rapidly after the collection at 4 °C and 2000 x g for 10 min. Then, the plasma was divided into 2.5-mL aliquots and transferred into 5-mL polypropylene screw top vials (Sarstedt). The plasma samples were snap-frozen in liquid nitrogen and stored at -80 °C until analysis. Additional 5 to 8 EDTA 9-mL plasma tubes were collected from 6 subjects in the same way to obtain a plasma pool. After centrifugation, the plasma of all 6 subjects was combined in a beaker glass, put on ice, and carefully homogenized on a magnetic stirrer for 5 min. Then, the plasma was transferred to 8-mL (Sarstedt) and 50-mL (Roth, Arlesheim, Switzerland) polypropylene vials with screw caps and aliquots of 5 mL and 20 mL stored at -80 °C until use.

Calibrators, quality control samples and spiked samples used for method validation were prepared freshly by spiking human pooled plasma with analytical standards of AEA, 2-AG and their deuterated analogues as internal standards (IS) before the extraction was performed, if not stated otherwise. For this purpose, 4 stock solutions of AEA were prepared by serial dilution in CAN at concentrations of 1 mg/mL as well as 100, 10, and 1 μg/mL. Three stock solutions with concentrations of 100, 10 and 1 μg/mL were prepared from 2-AG by serial dilution. Combined spiking solutions containing AEA and 2-AG at the same concentration used to freshly prepare calibrators and quality control samples were obtained from these stock solutions and referred to as spikers (1.0, 0.50, 0.35, 0.20, 0.10, 0.05, 0.035, 0.02 μg/mL). The 3 lowest concentrations were obtained by serial dilution from spikers with higher concentrations. Independent stock and spiking solutions were prepared for calibrators and quality control samples and stored in 2-mL brown glass vials with teflon-coated screw tops (Infochroma, Zug, Switzerland) at -20 °C. Individual dilutions of AEA-D4 and 2-AG-D5 in ACN were prepared (10 μg/mL) and combined to obtain a stock solution containing both IS at a concentration of 0.3 μg/mL. The IS solution was aliquoted (1 mL) and stored at -20 °C in teflon-coated screw top glass vials (Infochroma).

Liquid-liquid extraction of plasma samples was performed in 10-mL Pyrex glass tubes (Thermo Fisher Scientific, Basel, Switzerland), which were put on ice. The plasma was thawed in a water bath at 20 °C and put on ice thereafter. 1.5 mL of EDTA plasma was combined with IS (15 μL) and the samples mixed carefully by horizontal shaking for 10 s. For spiked plasma samples, such as calibrators and controls, 1.5 mL of the pooled plasma was used and mixed with 15 μL of IS and 15 μL of the appropriate spiking solution to obtain the target concentration. The samples were kept on ice for 30 min to release EC bound to proteins. Then, 3 mL of ice-cold toluene was added and the samples vortexed for 1 min. Extraction was performed at room temperature on a horizontal shaker (C. Gerhardt, Königswinter, Germany) with 90 oscillations/min. After centrifugation of the samples at 4 °C and 3000 x g for 10 min, the supernatant was transferred to a sample collection tube (Infochroma) and the extraction of the plasma repeated in the same manner with another 2 mL of ice-cold toluene. The organic phases of the 2 extracts were combined and the solvent was reduced to dryness under a gentle stream of nitrogen at 40 °C in a TurboVap® LV evaporator (Zymark, Oftringen, Switzerland). When the samples were completely dry, about 400 μL of hexane was added and the samples vortexed for 15 s before sonicating for 5 min. Then, the samples were transferred to a GC autosampler vial and the sample collection tubes rinsed with another 200 μL of hexane. The organic solvent was evaporated at 40 °C under nitrogen. Derivatization was performed directly in the GC autosampler vial with a 1:1 mixture of hexane – BSTFA/TMCS. The derivatization reagent was added (100 μL), the vial immediately closed with a teflon-coated crimp cap and vortexed before sonication for 3 min. Derivatization was performed for 30 min at room temperature. Then, the samples were dried under nitrogen to remove excess derivatization reagent and the residues dissolved in 25 μL of heptane, vortexed and sonicated for 3 min before injection into the GC/MS system.

**GC/MS analysis**

An Agilent 6890N gas chromatograph with an Agilent 7683 automatic liquid sampler and an Agilent 5975C mass selective detector (MSD) were used for the quantitation of AEA and 2-AG (Agilent Technologies, Palo Alto, CA, USA). Pulsed splitless injection mode with a pulse pressure of 30 psi for 1 min was used to inject 5 μL of the samples onto an Agilent DB-1 MS capillary column (30 m x 0.25 mm i.d.; 0.25-μm film thickness). After a 0.5-min column equilibration the following temperature program was applied: 150 °C for 1 min, 25 °C/min to 250 °C, 7.5 °C/min to 300 °C, 30 °C/min to 330 °C, and finally 330 °C for 3.5 min, resulting in a total run time of 16.2 min. Helium was used as carrier gas at a constant flow rate of 1 mL/min. The injector temperature was set to 250 °C. Mass spectral data were obtained with positive chemical ionization mode (PCI) using methane combined with selective ion monitoring (SIM). The MSD was operated between 9.0 and 16.2 min. Two groups of ions were recorded with low resolution, corresponding to AEA (9.0-10.7 min) and AG (10.7-16.2 min). The electron multiplier voltage was increased by 1200 V between 10.5 and 11.3 min. For AEA (AEA-D4) *m/z* 420.2 (*m/z* 424.2) was recorded as the quantifier ion, whereas *m/z* 330.2 (*m/z* 334.2) and *m/z* 448.2 (*m/z* 452.2) were monitored as qualifier ions. The dwell times were 100 and 40 ms for the quantifier and qualifier ions, respectively. The ion *m/z* 433.2 (*m/z* 438.2) was the quantifier of 1-AG and 2-AG (1-AG-D5 and 2-AG-D5) with the dwell time being 100 ms. As qualifier ions *m/z* 434.2 (*m/z* 439.2) and *m/z* 451.2 (*m/z* 456.2) were recorded with dwell times of 40 ms. The temperature settings of the MSD were 280, 250, and 150 °C for the transfer line, the ion source and the quadrupol, respectively. For instrument operation, data acquisition and analysis enhanced Agilent ChemStation software version E 01.00.237 was used.

**Calibration and quantitation**

2-AG is known to partly isomerize to the analytical artifact 1-AG during sample processing. Therefore, the concentration of AG is reported as the sum of 2-AG and 1-AG throughout this manuscript. Internal calibration based on peak area ratios of AEA, 2-AG and 1-AG *vs.* their respective deuterated IS was used for quantitation. As no blank matrix, i.e. plasma free of AEA and 2-AG, is available for the analysis of EC, the standard addition method was used to prepare calibrators, quality control samples, and spiked samples used for assay validation. Pooled plasma with unknown concentrations of AEA and 2-AG was used (referred to as blank plasma) and spiked with known concentrations of EC. Mean peak area ratios of AEA, 2-AG and 1-AG *vs.* the corresponding deuterated IS were determined in the blank plasma with each sample set (n = 3) and subtracted from the peak area ratios obtained after spiking to calculate corrected peak are ratios. Five calibration sets containing both EC were prepared and measured on 5 different days, covering 0.35 to 5 ng/mL for AEA and 1.0 to 10.0 ng/mL for 2-AG. Six calibrator levels were used for AEA (0.35, 0.5, 1.0, 2.0, 3.5, 5.0 ng/mL) and 5 for AG (1.0, 2.0, 3.5, 5.0, 10.0 ng/mL). Three blank plasma samples were freshly prepared for each calibration set, and the mean value of the peak area ratios used to subtract from the spiked samples (i.e. calibrators). A combined calibration curve was calculated from the mean values of the corrected peak area ratios of the 5 calibrators on each concentration level and was based on non-weighed linear regression analysis (least-squares model).

**Method validation**

The assay performance was characterized as precision, accuracy, linearity, limit of detection (LOD), lower limit of quantitation (LLOQ), and recovery of the extraction. In addition, pre-analytical stability of whole blood, long-term stability of frozen plasma samples, freeze-thaw stability and bench top stability of EDTA plasma samples and autosampler stability of derivatized samples were assessed. Intraday (n = 6) and interday (n = 6) data for precision and accuracy were obtained with spiked control samples.

The LOD was defined as S/N = 3 and assessed with a dilution series of spiked samples. The lowest concentration, which had a precision and accuracy (deviation of the spiked concentration from the target value) of ± 20 % was defined as LLOQ and was determined with spiked control samples (n = 6). Intraday and interday precision and accuracy data were assessed with 3 spiked concentrations for AEA (0.35, 0.7 and 3.0 ng/mL) and 2 concentrations for 2-AG (3.0 and 7.0 ng/mL). Six samples of each concentration were freshly prepared on the same day and on 6 different days for that purpose.

The extraction recovery in plasma was assessed with the deuterated standards of AEA and 2-AG due to the lack of blank plasma devoid of EC. Six pooled plasma samples were spiked with AEA and 2-AG to obtain concentrations of 3 ng/mL. Three of these samples were extracted as described above. Just before the addition of the derivatization reagent, deuterated AEA and 2-AG were added as IS corresponding to plasma concentrations of 3 ng/mL and the samples dried under nitrogen. Each of the derivatized samples was analyzed 3 times by GC/MS and the mean values of the peak area ratios of the deuterated and the non-deuterated EC (i.e. peak area of the deuterated EC divided by the area of the non-deuterated EC) referred to as 100 %. Before the extraction of the other 3 samples was conducted, deuterated AEA and 2-AG were added to the plasma at concentrations of 3 ng/mL each. Again, each sample was measured 3 times after extraction and derivatization and mean values of the peak area ratios of the deuterated and non-deuterated analytes calculated. The peak area ratios of the EC obtained with these samples were compared with those, where the IS was added after the extraction and the recovery calculated.

**Stability of AEA and AG in whole blood**

The stability of AEA and AG in whole blood was determined according to the following procedure: 3 blood samples were collected from 3 subjects in EDTA plasma tubes and put on ice. One tube from each subject was centrifuged 15 min after collection and the plasma snap-frozen and kept at -80 °C until analysis. A second tube from each of the subjects was centrifuged after 75 min and the last tube 195 min after the blood was collected. All plasma samples were snap-frozen immediately after centrifugation and stored at -80 °C. The concentrations of AEA and AG were determined *vs.* the time passed between blood collection and centrifugation of the whole blood.

**Benchtop stability**

Benchtop stability was assessed with control samples spiked with 3 ng/mL of AEA and 2-AG. Six samples were freshly spiked in pooled plasma. Three of them were extracted according to the SOP after 30 min incubation on ice, derivatized and analyzed. The other 3 samples were kept for 4 h on ice before the extraction and sample preparation were performed. Mean total EC concentrations, SD and RSD of AEA and AG were compared between the 2 groups of samples.

**Autosampler stability**

The autosampler stability was determined with extracted and derivatized spiked control samples at room temperature. For that purpose, 3 samples were prepared from pooled plasma and spiked with 3 ng/mL of AEA and 2-AG. The samples were measured immediately after extraction and derivatization and then kept for 45 h on the autosampler. Then, the samples were analyzed a second time and the total EC concentrations compared with those obtained after immediate analysis.

**Freeze-thaw stability**

Pooled blank plasma was used to assess freeze-thaw stability at 2 concentration levels. The non-spiked blank plasma was used for the low level and spiked with 3 ng/mL of both EC to monitor freeze-thaw stability with higher concentrations. Nine aliquots of each concentration were snap-frozen and stored at -80 °C until analysis. One day after collection of the plasma pool, 3 aliquots of both concentration levels were thawed in a water bath at 20 °C and kept on ice for 30 min after complete thawing. Then, the aliquots were stored again at -80 °C. After 24 h, 6 aliquots of each of the 2 concentration levels were thawed and treated in the same manner as for the first cycle and were finally refrozen at -80 °C. Half of these aliquots underwent the second cycle. Again 24 h later 9 aliquots of each concentration level were thawed in a water bath at 20 °C and kept on ice for 30 min after the samples were completely thawed. Then, all samples were prepared and analyzed according to the SOP. Among these aliquots 3 per each concentration underwent the third cycle, 3 the second cycle and 3 were only thawed once. This procedure allowed studying the effect of repeated freezing and thawing on total EC concentrations with 1, 2, and 3 cycles.

**Long term stability**

To measure the long term stability of frozen samples, non-spiked blank plasma was used to test low concentration levels, whereas pooled spiked plasma with 3 ng/mL of AEA and AG was used for higher concentrations. Three samples of each were extracted, derivatized, and analyzed before the plasma was frozen, referred to as time point 0 (t = 0). Aliquots of each were snap-frozen immediately after collection and stored at -80 °C. After 2 (t = 1) and 4 weeks (t = 2), 3 aliquots were thawed and the samples extracted and prepared according to the SOP. Then, mean total EC concentrations, SD and RSD were calculated.

**Influence of food intake**

To study the effect of recent food intake on EC plasma concentrations, plasma samples were collected from 5 subjects under fasting conditions according to the SOP. Additional plasma samples were collected from the same subjects 2 h after ingestion of a regular non-standardized lunch. Amount and type of food were not regulated. However, the intake of alcoholic beverages was not allowed.

**Statistical analyses**

SigmaPlot for windows software, Version 12.0 (Systat Software, San Jose, CA, USA) was used for graphical presentations and to perform statistical analyses. Data were checked for normal distribution applying the Saphiro-Wilk normality test and input groups were compared with the paired t-test or the Wilcoxon signed rank test.