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Comprehensive profiling of the human circulating endocannabinoid metabolome: clinical sampling and sample storage parameters

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Abstract

Background—Endogenous cannabinoid-receptor ligands (endocannabinoids) and over a dozen related metabolites now comprise the “endocannabinoid metabolome”. The diverse (patho)physiological roles of endocannabinoids, the predictive/diagnostic utility of systemic endocannabinoid levels, and the growing interest in endocannabinoid-related pharmacotherapeutics mandate a valid clinical protocol for processing human blood that does not jeopardize profiling of the circulating endocannabinoid metabolome.

Methods—We systematically evaluated the potential effect of pre-analytical variables associated with phlebotomy and sample handling/work-up on the human-blood endocannabinoid metabolome as quantified by state-of-the-art liquid chromatography-mass spectrometry.

Results—Neither subject posture during phlebotomy nor moderate activity beforehand influenced the blood levels of the 15 endocannabinoid-system lipids quantified. Storage of fresh blood at 4°C selectively enhanced ethanolamide concentrations artifactually without affecting monoglycerides and nonesterified fatty acids, such as arachidonic acid. In marked contrast, ethanolamides and monoglycerides remained stable through three plasma freeze/thaw cycles, whereas plasma arachidonic acid content increased, probably a reflection of ongoing metabolism.

Conclusions—Class- and compound-selective pre-analytical influences on circulating human endocannabinoid levels necessitate immediate plasma preparation from fresh blood and prompt plasma apportioning and snap-freezing. Repeated plasma thawing and refreezing should be avoided. This protocol ensures sample integrity for evaluating the circulating endocannabinoid metabolome in the clinical setting.

Keywords

endocannabinoids; human; liquid chromatography-mass spectrometry; plasma; sampling protocol

Introduction

The endocannabinoid system includes two main, G protein-coupled cannabinoid receptors (CB1 and CB2), synthetic enzymes, such as diacylglycerol lipase, deactivating enzymes, such as fatty acid amide hydrolase and monoacylglycerol lipase, and transporters. Each of these components is affected by or influences endogenous ligands termed “endocannabinoids”, ubiquitous signaling lipids eliciting central and peripheral effects (1–3). The first endocannabinoids, arachidonylethanolamine (“anandamide”, AEA) and 2-arachidonoylglycerol (2-AG), were identified in the early 1990s (1–3). Several others have been isolated subsequently, and new endocannabinoids and related precursors/metabolites probably remain to be discovered (1). Consequently, diverse carboxamide, ethanolamide, glycerol-ester, and biogenic amine/amino acids and the unsaturated fatty acids from which they are derived now comprise the complement of endogenous signaling lipids and related metabolites collectively termed the endocannabinoid metabolome.

In man and other mammals, constituents of the endocannabinoid metabolome support signaling within the central nervous system and periphery to maintain physiological functioning and homeostasis. Endocannabinoid tone is therefore stringently controlled, principally through the physiological balance between biosynthesis and inactivation. Acute alterations in steady-state endocannabinoid levels are usually transient responses to maintain or re-establish homeostasis, whereas more sustained changes may be pathogenic (3–11). Pharmacotherapeutic redress of pathological changes in the endocannabinoid metabolome may benefit maladies involving neurodegeneration (e.g., Alzheimer’s and Huntington’s diseases and amyotrophic lateral sclerosis), substance abuse, and metabolic derangements supported by obesity (5, 11–13). The diverse (patho)-physiological effects of endocannabinoid mediators across organ systems, the “on-demand” nature of their production, and their prompt enzymatic deactivation imply that circulating endocannabinoid levels have diagnostic/predictive clinical value. In both animal models and humans, alterations in circulating AEA and/or 2-AG levels have been associated with pain, neurodegenerative disorders, inflammatory conditions, and metabolic syndrome (12, 14–16). The facts that plasma AEA levels have been shown to correlate with depression in human females (17) and that increased plasma 2-AG has been advanced recently as a clinical biomarker of cardiometabolic risk in obese men (14) are two direct demonstrations of the diagnostic clinical relevance of the endocannabinoid metabolome. The influence of palmitoylethanolamine (PEA) and oleoylethanolamine (OEA) on pain, inflammation, immune function, and appetite (6, 9, 18–22) provides evidence that circulating endocannabinoids other than AEA and 2-AG also have important implications for human health and translational science.

The (patho)physiological relevance of endocannabinoid signaling and alterations in the endocannabinoid metabolome to the clinic is particularly well illustrated by human female reproductive health. Although it is well accepted that the phytocannabinoid Δ^9 -tetrahydrocannabinol may adversely affect reproductive events in female marijuana users, emerging evidence suggests that programmed changes in endocannabinoid tone help direct uterine and embryonic functions during pregnancy (11, 23, 24). Endocannabinoid effects on female reproductive status appear temporally and spatially coordinated throughout many stages of pregnancy and childbirth, and perturbations in endocannabinoid ratios/amounts can adversely affect childbearing (12, 24–33). In particular, programmed variations in AEA levels may reflect pleiotropic actions of this endocannabinoid critical to female reproduction and fertility (11, 13, 14). Whether variations in the levels of endocannabinoids other than AEA are determinants of pregnancy outcome remains an intriguing possibility, as does the potential therapeutic impact of endocannabinoid-related drugs on female reproductive

problems. For these reasons, clinical application of lipidomics to help define and quantify the impact of diverse endocannabinoids and their dynamics upon female reproductive events has been advocated (11), as well as providing hope that endocannabinoid signaling could be utilized in the correction of infertility (23).

Largely by historical precedent, method development in the endocannabinoid field has focused upon AEA and 2-AG quantification (9, 16). Temperature-related increases in brain 2-AG and AEA (10, 34) and blood AEA (35) *ex vivo*, as well as the reported increase in circulating AEA during exercise (36), suggest that both sample handling/storage and subject activity are important variables able to bias, if not confound, quantitative endocannabinoid profiling. Nonetheless, systematic study of the influence of pre-analytical factors on even 2-AG and AEA measurement in human blood is conspicuously lacking. Furthermore, the potential of pre-analytical factors to confound determination of sample endocannabinoid levels over the wide range of metabolites presently considered to comprise the endocannabinoid metabolome is unknown.

Our laboratory has recently developed and validated unique liquid chromatography-mass spectroscopy (LC-MS) methodology that enables, for the first time, the simultaneous, multiplex profiling of 15 known or implicated endocannabinoid signaling system lipids/metabolites presently considered to comprise the endocannabinoid metabolome (37, 38). In marked contrast to most analytical approaches (6, 10, 34–36, 39), these 15 analytes include not only AEA and 2-AG, but also related precursors/metabolites and members of several classes of lipid mediators that interact either directly or indirectly with the endocannabinoid signaling system. The LC-MS method incorporates internal standards for each analyte, resulting in improved quantitative accuracy (40), whereas previous methods either have relied upon limited internal standards for quantification or have restricted analysis to those endocannabinoid system constituents for which internal standards were available (41).

To enable future clinical studies on the pregnancy-related dynamics and therapeutic modulation of the human endocannabinoid system, we have defined the influence of diverse blood collection, processing, and handling parameters on the circulating endocannabinoid metabolome. Our results have allowed us to define the effects of sampling and sample processing/storage parameters on the comprehensive endocannabinoid metabolome and recommend clinical sampling and handling conditions that support the accurate quantitative profiling of the entire human-blood endocannabinoid metabolome, as currently appreciated.

Materials and methods

Reagents and standards

Fatty-acid free bovine serum albumin was purchased from EMD Chemicals (San Diego, CA, USA). Ethanol (200 proof), HPLC-grade acetone, and phosphate buffered saline were from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol and chloroform were purchased from Fisher Scientific (Pittsburg, PA, USA). High-purity nitrogen gas was purchased from Med Tech Gases (Medford, MA, USA). The endocannabinoid standard mixture of 15 analytes and the internal standard mixture of the corresponding 15 deuterated analytes (Table 1) were synthesized at the Center for Drug Discovery, Northeastern University (Boston, MA, USA) or purchased from Sigma-Aldrich, Cayman Chemical (Ann Arbor, MI, USA) or Nu-Check Prep (Elysian, MN, USA) and prepared as detailed previously (37, 38). Analytes were stored as solids at -80°C under argon (38).

Subjects

Healthy non-pregnant women of childbearing age and mixed race/ethnicity (18–35 years) recruited from the Storrs area, CT, USA, volunteered to participate in this study. Exclusion

criteria were a history of chronic hypertension, hyperlipidemia, renal or liver disease; thyroid disorder; psychiatric disorder; or medication for psychiatric disorder. Written informed consent was obtained from the nine subjects entered into the study prior to sample collection. Each subject was contacted 1 month after participation to confirm that she had not been pregnant during the study. The protocol was approved by the University of Connecticut and Northeastern University Human Subjects Review Committees.

Phlebotomy and plasma preparation

Venous blood (44 mL) was collected into EDTA-containing tubes through an indwelling catheter placed into the ante-cubital vein. Blood was either held on ice for a defined time period (see below) or immediately centrifuged for plasma preparation (2000×g, 10 min). The recovered plasma was apportioned without delay into 1-mL aliquots, frozen, and held at −80°C prior to analysis.

Variations in subject posture and physical activity

In total, nine female subjects engaged in each of the following activities/postures prior to phlebotomy: supine posture, upright seated posture, immediately after 10 min of walking at a moderate pace on a treadmill, and 15 min after resting from the walking exercise. In all cases, plasma was immediately separated from the collected blood by centrifugation and frozen at −80°C.

Blood storage variables

A portion of the blood drawn from supine subjects was placed in a 4°C refrigerator for 1, 2, 4, or 8 h prior to plasma isolation by centrifugation. Plasma was then immediately frozen at −80°C and thawed only once prior to processing and analysis.

Plasma storage variables

Replicate aliquots of plasma prepared immediately after blood draw from supine subjects were subjected to a maximum of three freeze (−80°C)/thaw cycles over the course of 1 day prior to a final freezing before analysis.

Endocannabinoid extraction from standard mixtures and human plasma

Endocannabinoids were extracted from all samples (calibration standards, reference extraction standards, and plasma) by a modified Folch method (38, 42). A known aliquot of endocannabinoid internal standard mixture was introduced into each plasma sample (400 µL plasma volume), and protein was precipitated with one volume of ice-cold acetone followed by centrifugation at 20,000×g for 5 min. Acetone was removed from the resulting supernatant under nitrogen. To the remaining supernatant, 100 µL phosphate buffered saline, 1 vol of methanol, and 2 vol of chloroform were added successively for lipid phase extraction. The resulting two phases were separated by centrifugation (20,000×g, 5 min), and the lower organic layer was recovered quantitatively and evaporated to dryness under nitrogen. Samples were reconstituted in 50 µL ethanol, vortexed, briefly sonicated, and centrifuged (20,000×g, 5 min) prior to analysis.

Liquid chromatography-mass spectroscopy analysis

Analysis of endocannabinoid-system constituents and their deuterated internal standards was performed as previously detailed using a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Electron, San Jose, CA, USA) with an Agilent 1100 HPLC front-end (Agilent Technologies, Wilmington, DE, USA) (37, 38). Separation of each analyte was achieved using an Agilent Zorbax SB-CN column (2.1×50 mm, 5-µm) and gradient elution with 10mM ammonium acetate (pH 7.3 using ammonium hydroxide, solvent A) and 100%

methanol (solvent B). Chromatography was optimized to separate the free fatty acids during the first 6 min of the run in negative ion mode followed by a switch to positive ion mode to detect the ethanolamide and glycerol derivatives during the latter 9 min. Eluted peaks were ionized via atmospheric pressure chemical ionization and detected by their respective selected reaction monitoring transitions (38).

Data analysis

The significance of the differences between means was determined by one-way analysis of variance followed by Dunnett's post-test using Prism software, Version 4 (GraphPad, San Diego, CA, USA).

Results

Endocannabinoid extraction efficiencies from human plasma and quantification of standards

The standard curve for each of the 15 endocannabinoid metabolome analytes was linear. All evidenced a regression value >0.98 , except the docosahexaenoic acid (DHA) and arachidonic acid (AA) plots, which had a linear regression value of 0.85. Extraction efficiencies from plasma were $>90\%$ for each analyte and its corresponding deuterated standard, consistent with our previously reported quantitative recoveries for endocannabinoid standards in solution (38).

Effect of posture and physical activity

Neither moderate physical activity preceding nor subject posture during phlebotomy significantly ($p>0.05$) influenced the free acid, glyceride, or ethanolamide concentrations in human plasma which had been prepared immediately from whole blood, frozen, and thawed just prior to processing and analysis. Consequently, the concentration of each of the 10 endocannabinoid analytes detected in human plasma under all four activity/posture conditions studied can be considered a steady-state concentration and may be expressed as a range over all nine subjects (Table 1).

Effect of whole-blood storage at 4°C

Allowing whole blood to stand at 4°C significantly elevated the concentrations of four of the six ethanolamides in a time-selective fashion (Figure 1). The artifactual increase in AEA was most acute and evident within 1 h. In contrast, blood storage at 4°C for up to 8 h had no significant effect on the steady-state concentrations of the glycerols or fatty acids that are components of the circulating human endocannabinoid metabolome. As compared to the posture and physical activity studies (above) wherein frozen plasma was prepared immediately after blood sampling (Table 1), the endocannabinoid concentrations in plasma held for 8 h at 4°C were as follows: AEA increased (0.63 ng/mL, $p<0.01$), PEA increased (3.79 ng/mL, $p<0.01$), OEA increased (3.43 ng/mL, $p<0.01$), docosahexaenoyl ethanolamine (DHEA) doubled (0.62 ng/mL, $p<0.05$), 2-AG was unchanged (11.44 ng/mL, $p>0.05$), 2-oleoylglycerol (2-OG) was unchanged (1.57 μ g/mL, $p>0.05$), 2-docosahexaenoylglycerol (2-DHG) was unchanged (102 ng/mL, $p>0.05$), AA was unchanged (448 ng/mL, $p>0.05$), DHA was unchanged (393 ng/mL, $p>0.05$), and eicosapentaenoic acid (EPA) was unchanged (50.3 ng/mL, $p>0.05$).

Effect of three freeze/thaw cycles

The ethanolamine and glycerol derivatives were stable through three freeze/thaw cycles. Although the fatty acids DHA and EPA were likewise stable, AA increased significantly at the third freeze/thaw cycle (Figure 2, $p<0.05$).

Discussion

Several compelling considerations led us to under-take this first systematic study of the effect of pre-analytical variables on human-blood endocannabinoid levels: the increasing focus on pharmacological modulation of endocannabinoid signaling for therapeutic gain, the (patho)physiological and diagnostic importance of circulating endocannabinoid tone, the routine nature of blood sampling in the clinical setting, and our recently introduced LC-MS procedure that enables, for the first time, comprehensive multiplex quantification of 15 endocannabinoid metabolome constituents (1, 5, 21, 22). Furthermore, laboratory reports that AEA and 2-AG concentrations increase in post-mortem brain tissue (10, 34) and a clinical report that blood AEA increases during exercise (36) suggest the potential for pre-analytical artifacts from tissue handling and subject activity in the determination of at least these two endocannabinoids. One human study seeking a correlation between depression and plasma AEA and 2-AG levels states that the samples were processed and frozen within 120 min (17), which may allow for temperature and time-dependant variations. These data led us to focus on these factors as potential influences on all endogenous metabolites presently considered to comprise the endocannabinoid metabolome in the context of clinical blood sampling and sample preparation/ storage. Our aim is to define reliable sampling and blood processing/storage parameters that obviate processing artifacts and thereby allow comprehensive quantification of the circulating endocannabinoid metabolome in human blood with state-of-the-art LC-MS methodology we have previously developed (37, 38).

We demonstrate that neither moderate physical activity prior nor postural position during blood collection influences the circulating endocannabinoid metabolome. These data constitute important information that can be communicated to human subjects regarding activities in which they can engage prior to blood sampling for circulating endocannabinoid quantification. Accordingly, it appears that normal daily activities may be conducted without artifactually compromising the blood endocannabinoid profile. Whereas Sparling et al. attributed the euphoric feeling that runners experience to increased circulating AEA (36), we did not observe a change in blood AEA (or any other circulating endocannabinoid) after moderate walking. The difference in activity level and its intensity between that study and ours – i.e., running for 50 min on a treadmill (36) vs. moderate walking for 10 min – may have contributed to the different response. The intriguing possibility of a relationship between blood endocannabinoid concentrations and graded physical activity is raised by this difference.

To determine the effect of post-phlebotomy sample handling on endocannabinoid concentrations, aliquots of blood collected from subjects in the supine position were held at 4°C for varying lengths of time prior to centrifugation and plasma isolation. Allowing whole blood to sit at 4°C increased the concentrations of all ethanolamides present, whereas glycerides and fatty acids were unaffected. The acute increases in six circulating ethanolamides we observed during blood storage at 4°C corroborate and greatly extend the observation (19) that erythrocyte AEA synthesis occurs *ex vivo* in blood held at 4°C.

To the best of our knowledge, our work with endocannabinoid standards validating the LC-MS procedure used herein (38) and this investigation with human plasma are the only studies addressing comprehensive endocannabinoid freeze/thaw stability. In plasma subjected to a maximum of three freeze/thaw cycles, the glycerides, ethanolamides, DHA, and EPA were stable, whereas AA increased significantly by the third freeze/thaw cycle. This observation, together with our finding that AA in an aqueous bovine serum albumin solution is freeze/thaw unstable (38), demonstrates the selective thermal sensitivity of the AA constituent of the endocannabinoid metabolome. Because bovine serum albumin is free of bioactive constituents (e.g., enzymes) found in plasma, it is tempting to speculate that the

increase in AA with repeated plasma freeze/thawing may reflect ongoing metabolism, as might the increased plasma ethanolamide concentrations following storage of fresh blood at 4°C. Although we observed that the six endocannabinoid metabolome ethanolamides are stable in human plasma over three acute freeze/thaw cycles, active catabolism may underlie the significant, yet highly variable, losses of the fatty ethanolamides AEA, PEA, and OEA upon analysis of human serum stored at –80°C for 2 months following a single freeze/thaw cycle (41).

In summary, post-collection handling and storage of blood samples can jeopardize accurate quantitative profiling of the circulating endocannabinoid metabolome. In clinical trials that involve endocannabinoid quantification in human blood, it is recommended that volunteers be in a comfortable posture for phlebotomy. The collected blood should be centrifuged for plasma separation immediately thereafter. The plasma should then be divided into aliquots without delay, snap-frozen, and kept at –80°C until analysis. Although routinely practiced and attractive from the standpoint of convenience, storage of plasma “on ice” or under refrigeration or subjecting plasma to repeated freeze/thaw cycles prior to analysis of the circulating endocannabinoid metabolome must be avoided. These sampling and sample handling parameters should facilitate reliable clinical profiling of the circulating endocannabinoid metabolome in health and disease, thus supporting the intensifying quest for cannabinomimetic agents with translational and therapeutic potential (11, 12, 43).

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Figure 1. Ethanolamide concentrations were assessed for their stability at 4°C

Whole blood, taken when the subject was lying down, was centrifuged for plasma and allowed to sit at 4°C for 0, 1, 2, 4, or 8 h, and then frozen. Values were transformed to their logarithms so that their variances would be more Gaussian. (A) Anandamide (AEA) concentrations significantly ($p<0.05$) increased in the plasma after 1 h. (B) Palmitoylethanolamine (PEA) significantly ($p<0.01$) increased after 4 h. (C) Oleoylethanolamine (OEA) concentrations significantly ($p<0.01$) increased after 2 h. (D) Docosahexaenylethanolamine (DHEA) increased significantly ($p<0.05$) after 8 h.

Figure 2. Fatty acid concentrations were assessed in human plasma for their stability through three freeze/thaw cycles

Blood samples were taken from nine supine female volunteers and either frozen until analysis or subjected to up to three freeze/thaw cycles. (A) Arachidonic acid (AA) increased significantly ($p<0.05$) compared to the control sample at the third freeze/thaw cycle. No significant change was observed for (B) docosahexaenoic acid (DHA) or (C) eicosapentaenoic acid (EPA).

Table 1

Endocannabinoid standard mixture of 15 analytes, their abbreviations, and their steady-state concentrations in human plasma

Analyte	Abbreviation	Plasma concentration (range, ns ⁹), ng/mL
Arachidonylethanolamine (anandamide)	AEA	0.18–0.24
Palmitoylethanolamine	PEA	1.8–2.1
Oleoylethanolamine	OEA	1.3–1.4
Docosahexaenoyl ethanolamine	DHEA	0.31–0.34
Eicosapentaenylethanolamine	EPEA	n.d.
Eicosanylethanolamine	EEA	n.d.
2-Arachidonoylglycerol	2-AG	9.9–11.0
2-Palmitoylglycerol	2-PG	n.d.
2-Oleoylglycerol	2-OG	1.2–1.5
2-Docosahexaenoylglycerol	2-DHG	75.5–90.4
2-Eicosapenaenoylglycerol	2-EPG	n.d.
2-Eicosanoylglycerol	2-EG	n.d.
Arachidonic acid	AA	472–574
Docosahexaenoic acid	DHA	328–388
Eicosapentaenoic acid	EPA	42.8–52.1

n.d., not detected.