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DOI: https://doi.org/10.1016/j.jchromb.2014.11.001

A quantitiative LC-MS/MS method for the measurement of arachidonic acid, prostanoids, endocannabinoids, *N*-acylethanolamines and steroids in human plasma

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Abbreviations

AA, arachidonic acid; ACN, acetonitrile; AEA, anandamide; ALDO, aldosterone; 2-AG, 2-arachidonoyl glycerol; A-GABA, *N*-arachidonoyl-gamma-aminobutyric acid; A-GLY, *N*-arachidonoyl-glycine; A-SER, *N*-arachidonoyl-serine; COR, cortisol; COX-2, cyclooxgenase-2; DAG, diacylglycerol; DHEA, dehydroepiandrosterone; ECs, endocannabinoids; EtOH, absolute ethanol; FBS, fetal bovine serum; IS, internal standard; LEA, linoleoyl ethanolamide; LLOQ, lower limit of quantification; LOD, limit of detection; CH_3OH, methanol; MRM, multiple reaction monitoring; NADA, *N*-arachidonoyl dopamine; NAPE, N-acylphosphatidylethanolamine; NE, noladin ether; OEA, oleoyl ethanolamide; PEA, palmitoyl ethanolamide; PGE_2, prostaglandin E_2; PGE_2-EA, prostaglandin E_2 ethanolamide; PMSF, phenylmethylsulfonyl; PROG, progesterone; SPE, solid phase extraction; TEST, testosterone; THB B_2, thromboxane B_2; SEA, stearoyl ethanolamide; UFLC, ultra-fast liquid chromatography; VIR, virodhamine

Abstract

Free arachidonic acid is functionally interlinked with different lipid signaling networks including those involving prostanoid pathways, the endocannabinoid system, *N*-acylethanolamines, as well as steroids. A sensitive and specific LC-MS/MS method for the quantification of arachidonic acid, prostaglandin E_2, thromboxane B_2, anandamide, 2-arachidonoylglycerol, noladin ether, lineoyl ethanolamide, oleoyl ethanolamide, palmitoyl ethanolamide, steroyl ethanolamide, aldosterone, cortisol, dehydroepiandrosterone, progesterone, and testosterone in human plasma was developed and validated. Analytes were extracted using acetonitrile precipitation followed by solid phase extraction. Separations were performed by UFLC using a C18 column and analyzed on a triple quadrupole MS with electron spray ionization. Analytes were run first in negative mode and, subsequently, in positive mode in two independent LC-MS/MS runs. For each analyte, two MRM transitions were collected in order to confirm identity. All analytes showed good linearity over the investigated concentration range ($r > 0.98$). Validated LLOQs ranged from 0.1 to 190 ng/mL and LODs ranged from 0.04 to 12.3 ng/mL. Our data show that this LC-MS/MS method is suitable for the quantification of a diverse set of bioactive lipids in plasma from human donors (n=32). The determined plasma levels are in agreement with the literature, thus providing a versatile method to explore pathophysiological processes in which changes of these lipids are implicated.

Keywords

LC-MS/MS; arachidonic acid, endocannabinoids; lipids; steroids; *N*-acylethanolamines; palmitoylethanolamide; prostanoids, noladin ether

1. Introduction

Arachidonic acid (AA) is not only an abundant component of cell membranes (incorporated into phospholipids), but also is the precursor of prostanoids and all endocannabinoids (ECs). Thus, AA is of primary importance in neuro- and immunomodulation, energy metabolism, as well as the cardiovascular system [1–3]. The pool of free AA, which may vary in different tissues and between individuals, is known to be regulated by phospholipase A2 (PLA2) and 2-arachidonoyl glycerol (2-AG) hydrolysis. Recently, the levels of 2-AG and AA were shown to inversely correlate with the free AA generated upon hydrolysis of 2-AG which was shown to act as precursor for eicosanoid biosynthesis [4]. Prostanoids are a family of lipid mediators that play key roles in both inflammatory and neuropsychiatric processes. They are produced upon the enzymatic oxygenation of AA by cyclooxygenase-2 (COX-2) resulting in e.g. the production of prostaglandin E_2 (PGE_2) and thromboxane B_2 (THB B_2) [5]. Attention has recently been paid to the fact that like AA, ECs containing an arachidonoyl moiety are good substrates of COX-2, leading to the formation of similar prostanoid derivatives (e.g., prostaglandin E_2 ethanolamide (PGE_2EA)) [6].

The endocannabinoid system is involved in different physiological and pathophysiological processes that occur mainly in the central nervous system (CNS) and immune system, but also exerts regulatory effects on metabolic processes and vascular tone [7]. ECs are produced “on demand” in the body from precursor phospholipids containing arachidonyl moieties derived from arachidonic acid including *N*-acylphosphatidylethanolamine (NAPE) and diacylglycerol (DAG) [8,9]. These bioactive lipids are not stored in vesicles, but are transported across the plasma membrane by as yet poorly understood mechanisms [10]. The distribution of ECs between tissues and plasma is not equal and a correlation between brain and plasma levels cannot be drawn, unless experimentally shown. By definition, ECs interact with both central (CB_1) and peripheral (CB_2) G protein-coupled cannabinoid receptors and act as partial or full agonists [9]. The most important and best studied ECs are anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), which can be found in numerous tissues [9,11]. In addition to the classical ECs, virodhamine (VIR), noladin ether (NE) and *N*-arachidonoyl dopamine (NADA) have been proposed to act as ECs

[12]. However, only limited information is available about the quantifiable amounts of these minor ECs in plasma and tissues. Another class of related compounds are amino acid conjugates (e.g., *N*-arachidonoyl-gamma-aminobutyric acid (A-GABA), *N*-arachidonoyl-glycine (A-GLY) and *N*-arachidonoyl-serine (A-SER)), which have also been proposed to exert physiological effects [13,14]. Furthermore, *N*-acylethanolamines other than AEA, which exhibit distinct saturated or unsaturated fatty acids and show no (or little) direct effect on CB receptors (i.e., linoleoyl ethanolamide (LEA), palmitoyl ethanolamide (PEA), oleoyl ethanolamide (OEA), stearoyl ethanolamide (SEA)), have been shown to act as ECs entourage molecules, possibly acting in concert with ECs [15–18]. Yet another important class of signaling lipids involves the steroids, which appear to functionally interlink the different AA lipid signaling networks, including the endocannabinoid system [19,20]. Steroids (glucocorticoids and mineralocorticoids) are involved in numerous physiological processes, including immunomodulation [21]. They are generated from cholesterol in the adrenal glands or the gonads, but some can also be formed in the CNS (neurosteroids) [22,23].

LC-MS/MS is a useful method for the quantitative analysis of lipophilic and apolar metabolites because it allows short analysis time, complete automation, minimal sample preparation (no derivatization), and it is applicable to analytes with a broad range of molecular masses and polarities. It has been widely used for the analysis of ECs and similar lipids [24–28]. However, it has only recently been used for the analysis of steroids [29–31].

To our knowledge, this is the first time that an LC-MS/MS method has been developed to analyze a combination of different biologically important lipids, including diverse arachidonic acid metabolites, *N*-acylethanolamines and steroids simultaneously in human plasma. The development of a sensitive analytical method to measure a wide range of lipids at physiological concentrations is required to evaluate the role of these metabolites and their possible cross talk in different diseases. In this study, we report a fully validated analytical LC-MS/MS method that first allows the simultaneous measurement of AA, PGE_2 and THB B_2 in the negative mode and, subsequently, AEA, 2-AG, aldosterone (ALDO), cortisol (COR), dehydroepiandrosterone (DHEA), LEA, NE,

OEA, PEA, progesterone (PROG), SEA and testosterone (TEST) in the positive mode in two LC-MS/MS runs.

2. Materials and methods

2.1. Materials

Analytical and internal standards were purchased from Cayman Chemical, Tallinn, Estonia (arachidonic acid (AA or 5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid); AA-d_8 (5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic-5,6,8,9,11,12,14,15-d_8 acid); anandamide (AEA or *N*-(2- hydroxyethyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide); AEA-d_4 (*N*-(2- hydroxyethyl-1,1,2,2-d_4)- 5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide); 2-arachidonoyl glycerol (2-AG or 5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid, 2-glyceryl ester); 2-AG-d_5 (5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid, 2-glyceryl-1,1,2,3,3-d_5 ester); *N*-arachidonoyl glycine (A-GLY or *N*-[1-oxo-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenyl]-glycine); *N*-arachidonoyl-L-Serine (A-SER or N-[1-oxo-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenyl]-L-serine); linoleoyl ethanolamide (LEA or *N*-(2- hydroxyethyl)-9*Z*,12*Z*-octadecadienamide); LEA-d_4 (*N*-(2-hydroxyethyl-1,1,2,2-d_4)-9*Z*,12*Z*-octadecadienamide); *N*-arachidonoyl dopamide (NADA or *N*-(2,3-(4-dihydroxyphenyl)ethyl- 5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide); noladin ether (NE or 5Z,8Z,11Z,14Z-eicosatetraen-2-glyceryl ether); oleoyl ethanlonamide (OEA or *N*-(2-hydroxyethyl)-9*Z*-octadecenamide); OEA-d_4 (*N*-(2-hydroxyethyl-1,1,2,2-d_4)-9*Z*-octadecenamide); palmitoylethanolamide (PEA or *N*-(2-hydroxyethyl)-hexadecanamide); PEA-d_5 (*N*-(2-hydroxyethyl)-hexadecanamide-15,15,16,16,16-d_5); prostaglandin E_2 (PRE_2 or 9-oxo-11*α*,15S-dihydroxy-prosta-5Z,13E-dien-1-oic acid); PGE_2-d_4 (9-oxo-11*α*,15*S*-dihydroxy-prosta-5*Z*,13*E*-dien-1-oic-3,3,4,4-d_4 acid); prostaglandin E_2 ethanolamide (PGE_2EA or *N*-(2-hydroxyethyl)-9-oxo-11*α*,15*S*-dihydroxy-prosta-5*Z*,13*E*-dien-1-amide); stearoyl ethanolamide (SEA or *N*-(2-hydroxyethyl)-octadecanamide); thromboxane B_2 (THB B_2 or 9*α*,11,15*S*-trihydroxythromba-5*Z*,13*E*-dien-1-oic acid); virodhamine (VIR or O-(2-aminoethyl)-5Z,8Z,11Z,14Z-eicosatetraenoic acid, ester, monohydrochloride)); from Sigma-Aldrich, Steinheim, Germany (aldosterone (ALDO or 11*β*,21-dihydroxypregn-4-ene-3,18,20-trione); androsterone (ANDRO or 3*α*-hydroxy-5*α*-androstan-17-one); hydrocortisol or

cortisol (COR or 11*β*,17*α*,21-trihydroxypregn-4-ene-3,20-dione); *trans*-dehydroepiandrosterone (DHEA or 3-*β*-hydroxy-5-androsten-17-one); ganaxolone (GANA or 3*α*-hydroxy-3*β*-methyl-5*α*-pregnan-20-one); progesterone (PROG or 4-pregnene-3,20-dione); PROG-d_9 (progesterone-2,2,4,6,6,17*α*,21,21,21-d_9); tetrahydrodeoxycorticosterone (THDOC or 3*α*,21-dihydroxy-5*α*-pregnan-20-one) and allopregnanolone (THP or 5*α*-pregnan-3*α*-ol-20-one)), from Fluka (testosterone (TEST or 17*β*-hydroxy-4-androsten-3-one)); from Alsachim, Illkirch Graffenstaden, France (COR-d_4 and TEST-d_4); and from TOCRIS (*N*-arachidonyl-gamma-aminobutyric acid (A-GABA or 4-[[(5*Z*,8*Z*,11*Z*,14*Z*)-1-Oxo-5,8,11,14-eicosatetraenyl]amino]butanoic acid)). HPLC-grade methanol (CH_3OH), HPLC-grade acetonitrile (ACN), ammonium acetate, formic acid and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma-Aldrich, Steinheim, Germany. HPLC-grade ethyl acetate was obtained from Acros Organics, New Jersey, USA. Absolute ethanol (EtOH) was obtained from the Federal Swiss Government. Deionized water (18.2 MΩ x cm) was obtained from an ELGA Purelab Ultra Genetic system (VWS (UK) Ltd, ELGA LabWater, UK). AQUASIL used for silanization was purchased from Thermo Scientific.

2.2. Silanization of glass and plastic ware

To prevent absorption of lipids, glass tubes and plastic tips were immersed for 2-5 min on AQUASIL solution (20 mL in 1L of distilled water), rinsed with methanol and dried at room temperature prior to the analysis.

2.3. Standard solutions

In order to prepare the standard mixtures used as calibrators (calibration solutions) and internal standards (IS), solutions of 1.0 and 0.01 mg/mL of analytical and labeled standards were prepared in EtOH. These solutions were kept at -80°C and the stability of the analytes was monitored throughout the analysis. The concentrations used for preparing calibrators and IS mixtures were selected according to the expected concentration of analytes in the plasma as well as their analytical limit of detection (LOD). Calibrators were divided in four concentration groups: The first group included AEA, LEA, NADA, PROG, TEST and VIR, in a calibration range from 0.04-50 ng/mL.

The second group contained 2-AG, A-GABA, A-GLY, ALDO, A-SER, NE, OEA, PEA, PGE_2, SEA and THB B_2 in a calibration range from 0.08-100 ng/mL. The third group included ANDRO, COR, DHEA, PGE_2EA, THDOC and THP in an analytical range from 0.4-500 ng/mL. The fourth group contained AA in an analytical range from 12.3-15000 ng/mL. The concentrations of the labeled standards and GANA used as internal standards were: 20 ng/mL for AEA-d_4, LEA-d_4, OEA-d_4, PEA-d_5, PGE_2-d_4, PROG-d_9 and TEST-d_4; 50 ng/mL for 2-AG-d_5; 150 ng/mL for COR-d_4; 400 ng/mL for GANA; and 1500 ng/mL for AA-d_8. In order to achieve these analytical concentrations in a final volume of 100 µL, 10 µL of a tenfold concentration of the calibrator and IS mixture were used (10 µL of 2-AG-d_5 500 ng/mL solution, brought upto a final volume of 100 µL to achieve a concentration of 50 ng/mL). The calibration curve used for the analysis of plasma samples was built to include 6-9 calibration points.

2.4. Plasma samples

For the validation, pooled plasma purchased from the blood bank in Bern (Blutspendezentrum SRK Bern) was used. Aliquots of 0.5 mL were used to construct calibration curves and prepare quality control samples (QC). QC (n=6) were prepared to evaluate intra- and inter-day levels of precision (non-spiked samples (QC_0)), and to evaluate the efficiency of analyte recoveries at low (QC_4: level 4) and high (QC_7: level 7) concentrations (see Table 4)). QC were prepared and stored at -80°C until analysis. QC used for the analysis of inter-day precision, recovery and stability were run over 4 different days by two different analysts within a period of 4 weeks. The plasma used for preparing QC for intra-day analysis came from a different pool from the plasma used for preparing QC used for the inter-day evaluation. QC samples were prepared as follows: 1) Frozen plasma was thawed for the analysis of intra-day variability (1 freeze/thaw cycle). 2) Plasma was thawed/aliquoted to prepare QC for the inter-day analysis (QC_0, QC_4 and QC_7) and then frozen/thawed for the measurement of QC_0, QC_4 and QC_7 for the inter-day analysis (2 freeze/thaw cycles).

The quantities of arachidonic acid, prostanoids, ECs, EC-related lipids and steroids were measured in the plasma of 32 healthy male volunteers as part of a study performed at the Department of Psychiatry, Psychotherapy and Psychosomatics,

Psychiatric Hospital of the University of Zurich. The study was approved by the Ethics Committee of the Canton Zurich and all participants gave written informed consent before inclusion in the study. Blood was collected in 10 mL BD Vacutainer K2EDTA tubes for plasma (Vacutainer Systems, becton Dickinson, Plymouth, UK) and spiked with 10 µL of PMSF solution in ethanol into 1 mL of blood (final 1 mmol/L PMSF to protect against enzymatic activity by serine proteases). Plasma was obtained ca. 3 hours after blood collection by centrifuging at 1,300 g for 10 min at 4°C. Blood samples were kept on ice until plasma generation. Plasma (ca. 5mL) was stored at -80°C prior the analysis.

2.5. Sample preparation

Plasma was thawed inside the refrigerator at 6°C for 3-4 hours prior the analysis (under our working conditions, 3 hours is the minimal time required for total thawing). In a 2 mL Eppendorf tube containing 1 mL ACN with 0.1 % formic acid, 10 µL of IS solution (see section 2.3, preparation of standard solutions) was spiked. The solution was kept at -20°C for 30-60 min prior to processing. Using silanized pipette tips, 0.5 mL plasma was added to the spiked ACN solution. The mixture was thoroughly vortexed and placed inside the refrigerator (6°C) for 10 min to facilitate the protein precipitation process. Subsequently, the sample was centrifuged at 16,100 g at 4°C for 5 min. The supernatant was transferred to a silanized glass tube, diluted with 9 mL water and extracted by solid-phase separation (C18 Sep-Pak cartridge fromWaters AG Baden-Dättwil, Switzerland, pre-activated with 3 mL CH_3OH and equilibrated with 3 mL 10% ACN). Cartridges were washed with 3 mL 10% ACN and eluted with 3 mL ACN/ethyl acetate (1:1). The eluates were evaporated to dryness under nitrogen. The samples were reconstituted in 100 µL ACN (final volume) and centrifuged for 5 min at 16,100 g at 4°C. Carefully, 80 µL were pipetted out, placed into conic amber vials and analyzed by LC-MS/MS (10 µL injection volume).

2.6. Chromatographic and mass spectrometric conditions

2.6.1. Equipment

Analyses were conducted on an LC-MS/MS system consisting of an API 4000 QTrap mass spectrometer equipped with a TurboIonSpray probe (AB Sciex Concord, Ontario, Canada) connected to a Shimadzu UFLC (Shimadzu Corporation, Kyoto, Japan). Data acquisition and analyses were performed using Analyst software version 1.5 (AB Sciex Concord, Ontario, Canada).

2.6.2. Chromatographic conditions

Analytical LC separations were performed on a Reprosil-PUR C18 column (3 µm particle size; 2×50 mm, Dr. A. Maisch, High Performance LC-GMBH, Ammerbuch, Germany) with a flow rate of 0.35 mL/min and oven temperature of 40°C using a gradient of CH_3OH containing 2 mM ammonium acetate (eluent B) and water containing 2 mM ammonium acetate and 0.1% formic acid (eluent A). The gradient was as follows: 15% eluent B for 0.5 min; 15-70% B from 0.5 - 3.5 min; 70-99% B from 3.5-8.0 min and held at 99% from 8.0-11.0. From 11.0-11.5 min, the column was re-equilibrated to 15% B and conditioned from 11.5-13 min at 15% B. The autosampler was cooled at 4°C.

2.6.3. MS/MS detection

The TurboIon Spray interface was operated in negative ionization mode for the analysis of AA, PGE_2 and THB_2 and in positive mode for the analysis of AEA, 2-AG, A-GABA, A-GLY, ALDO, ANDRO, A-SER, COR, DHEA, LEA, NADA, NE, OEA, PEA, PGE_2-EA, PROG, SEA, TEST, THDOC, THP and VIR. The parameters of the source using nitrogen as a curtain gas were as follows: capillary ion spray voltage +4500 V in positive and -4250 V in negative modes, respectively; temperature 600 °C; curtain gas 25 psi, GS1 50 psi and GS2 50 psi. The entrance potential and collision cell exit potentials were both set to 10 V. The analytes were measured in the multiple reaction monitoring mode (MRM). Two MRM, one quantifier (Q) and one qualifier (q) were considered in the analysis. The MRM parameters (retention time, precursor ion/ product ion, declustering potential, collision energy) for the quantifier MRM used in the survey and the respective internal standard (IS) are presented in Table 1. For the negative ionization mode, a dwell time of 50 ms was used for all MRM transitions, while for the positive ionization mode, the schedule MRM function was applied (target scan time of 0.8 sec and MRM

detection window of 100 sec). Data were acquired and processed using Analyst software.

2.7. Validation

The method was validated using external calibrations spiking calibration solutions (see section 2.3) into plasma and processing following section 2.5 on 6 consecutive days (n=6). Quality control samples (*n*=6 of QC_0, QC_4 and QC_7) were measured to fully validate the method (see section 2.4). The results were analyzed based on the peak area ratio between the analyte and IS. The IS used for each analyte measured are listed in Table 1. The full validation of the method was performed only for the analytes that could be detected in human plasma (AA, PGE_2, THB B_2, AEA, 2-AG, LEA, NE, OEA, PEA, SEA, ALDO, COR, DHEA, PROG and TEST). However, MRM, chromatographic conditions, LOD and low limit of quantification (LLOQ) of the analytes evaluated (except for VIR, which could not be recovered after spiking) were determined and are presented in Tables 1 and 2. Calibration curves were generated by subtracting the endogenous amount of an analyte from the spiked amount (blank subtraction). The results of the analysis allowed the determination of LOD, LLOQ, accuracy, linearity, precision and stability. The effect of the matrix on the analytes will be also discussed. Additionally, the levels of the compounds investigated were analyzed in 32 healthy male volunteers (see section 2.4).

3. Results

3.1. LOD/LLOQ

Due to the endogenous presence of the validated analytes in plasma, the instrumental LOD (signal-to-noise ratio 3) of standards was used (Table 2). The LLOQ was obtained from spiked plasma as the concentration at which the signal-to-noise ratio was 10 and showed positive values after blank subtraction (the area ratio of a spiked analyte in plasma subtracting the area ratio of the analyte in blank plasma). For the analytes not detected in plasma, the LOD and LLOQ (Table 2) were determined as explained above

for the LLOQ of the endogenous substances (signal-to-noise ratio 3/10 for the LOD/LLOQ).

3.2. Accuracy and linearity

The accuracy (percentage ratio of the measured concentration and theoretical concentration) was determined at three concentrations (see Table 3). Samples were prepared by spiking the different calibration solutions together with the IS prior the addition of plasma (in a similar way as was used for the preparation of the calibration curves) and subsequent processing as described in section 2.5. After subtraction of the blank, the accuracy was determined. Quantification was performed using linear regression from calibration curves (ratio of peak area analyte/ peak area IS vs. analyte concentration) using 6-9 calibration points (see section 2.3). For all the analytes validated, the concentration range displayed directly proportional results (n=3) indicating that the method is linear. In Table 3, the results of accuracy and linearity are summarized.

3.3. Precision, stability and recovery

The precision (standard deviation) of the method was determined using quality control samples QC (see section 2.4) and the results are summarized in Table 4. The low intra-day variability reflects the high performance of the method. All analytes show variation coefficients (CV) below 15%, except for AEA (20%). The inter-day analyses performed at three different concentrations (QC_0, QC_4 and QC_7 (see section 2.4)) not only helped to establish the precision of the method, but also the stability (within 1 month). The highest variability is observed in QC_4. This could be explained by the fact that the amounts of analytes spiked are low as compared to the endogenous levels of the analytes (the variability is less noticeable when spiking samples with higher concentrations of analytes, such as QC_7). This can be better appreciated when comparing the concentration spiked in QC_4 (Table 4) with the values reported in Table 5 (i.e., dynamic range and average of the analytical concentration obtained from the analysis of 32 plasma samples from healthy male volunteers (see section 2.4). Considering the complexity of the plasma as a matrix, the results obtained for precision

are acceptable (see Table 4) and show that the samples are stable under the experimental conditions used here. The recovery, calculated at two concentrations (QC_4 and QC_7), is within a conventional range of 70-130% (see Table 4). Recoveries were calculated after subtraction of blanks (average values of QC_0) of spiked QC samples (average values of QC_4 and QC_7). Interestingly, a significant difference existed between the average values of intra-day and inter-day analyses for 2-AG and TEST (see Table 4). As explained in section 2.3 (plasma samples), two different pool of plasma were used for the respective analyses.

3.4. Matrix effect

The assessment of the combined effect of the sample matrix and variable recoveries can be determined when evaluating the variability of the slopes (Table 3). Low variability (CV %) indicates that the precision and accuracy of the method are not affected by the matrix or by the workup. Additionally, to evaluate the matrix effect, absolute area values from calibration curves were used. Data obtained from calibrators spiked into solvent and processed (without matrix) and calibrators spiked in plasma (with matrix) were evaluated. We selected two subsequent arbitrary points inside the calibration curve and subtracted the area values to calculate a delta area. In theory, the delta area should be similar if a matrix effect does not exist. This is the case for most of the analytes (ALDO, COR, DHEA, OEA, PEA, PROG and TEST). However, the delta area is lower for AA, NE, PGE_2 and THB B_2, and higher for AEA, 2-AG and LEA, from which we conclude that a matrix effect for these analytes exists.

3.5. Application of the method

The levels of AA, AEA, 2-AG, ALDO, COR, DHEA, LEA, NE, OEA, PEA, PGE_2, PROG, SEA, TEST, and THB B_2 in human plasma were analyzed in 32 healthy male volunteers (A-GABA, A-GLY, ANDRO , A-SER, NADA, PGE_2EA, THP, and THDOC were not detected). Mean plasma concentrations are reported in Table 5 together with their respective mean analytical concentrations. Additionally, values previously described in literature are also reported.

4. Discussion

In an earlier study, we reported the results of an analysis of AA, 2-AG, AEA, OEA, PEA and PGE_2 in fetal bovine sera (FBS) using a quantitative GC-MS method [11]. The extraction procedure used for FBS analysis was the starting point for the current LC-MS/MS method validation in human adult plasma. The Folch extraction (as used in GC-MS analysis of FBS) followed by SPE clean up did not allow the efficient recovery of PEA and SEA in plasma samples of humans or rodents. Therefore, we precipitated plasma proteins using ACN or cold acetone, as previously reported [26,27,32]. However, the use of cold acetone led to lower recoveries of the steroids measured (data not shown). Consequently, we selected ACN precipitation, followed by SPE clean up, prior to LC-MS/MS analysis (see section 2.5). Interestingly, publications citing other methods of extraction for *N*-acylethanolamides including PEA (e.g., chloroform/methanol/Tris-HCl 50 mM pH 7.5 (2:1:1, v/v) [33], ethyl acetate/*n*-hexane (9:1 v/v) [34], toluene [35], or directly using SPE (Oasis HLB) and elution with ACN [16]), did not describe any problems in their analyses. We evaluated the Folch extraction method and experienced difficulties only during the quantitative extraction of PEA and SEA in plasma - not in FBS - using both GC-MS and LC-MS/MS (data not shown). The reason for this remains unclear, but we assume that strong plasma protein (e.g., albumin) binding may have contributed to these difficulties.

Originally, it was our intention to measure all analytes in a single injection. However, under the experimental conditions used this was not possible. Although the positive mode was the most sensitive mode for most of the compounds, AA, PGE_2 and THB B_2 could not be analyzed in this mode [24,36–38]. Only one publication measured AA in positive mode (using a Micromass Quatro Micro spectrometer from Waters) [39], but these results could not be reproduced using our instrument. Nevertheless, by performing two subsequent injections (first in negative and then in positive mode), inserting blank runs in between, we were able to quantitate all compounds.

With respect to the chromatography (see Figure 2 and supplementary Figure 1), all the analytes appeared as single peaks except for 2-AG (and its isomer 1-AG), 2-AG-d_5, THB B_2, and DHEA, which showed two peaks. In these cases, the two peaks were

integrated together for their analysis. Figure 2 shows chromatograms of the compounds validated with the presented method (standards and plasma).

To assess the reliability of the method, we compared the values obtained from healthy male volunteers with similar results previously reported in literature (see Table 5). In some cases, it was not possible to find information about the levels of the analytes studied only in males and, therefore, levels for both males and females or only females are presented in Table 5. The results obtained for AA, AEA, 2-AG, COR, DHEA, PROG and TEST are in full agreement with values reported in the literature [24–27,40–44]. The plasma levels of PROG show a high level of fluctuation in females, which is dependent on the age and stage of reproductive cycle [31]. This fluctuation has also been reported for AEA [28] although it remains questionable whether small AEA variations are physiologically meaningful. COR is a stress marker in humans and, therefore, the levels in plasma are known to oscillate under conditions of stress like participating in an experiment.

The values of OEA, PEA and SEA reported here are slightly higher than the values appearing in the literature. Both studies used for comparison only reported the gender, age and health status of the donors, but did not give information about the collection procedure or treatment of the samples prior to the analysis [26,27]. Most importantly, the use of PMSF blocks the enzymatic degradation of *N*-acylethanolamines by serine hydrolases, which may be reflected in the higher values obtained in the present study. In the case of LEA, no information could be found with regard to endogenous levels in human plasma, although we estimate that it is between 0.5-23 pmol/mL, according to information stated elsewhere [45].

NE was originally isolated from pig brain and described as an endocannabinoid in 2001 [46]. Subsequently, it was reported only once more from rat brains and peripheral tissues [47]. NE has, however, never been reported in human plasma. In our study, we could detect NE in human plasma (data not shown). This finding requires further validation using high-resolution MS and additional experiments with blood samples from other mammals, which goes beyond the description of the method reported here. Interestingly, two sensitive methods have been validated for the measurement of NE in

human plasma, but neither of them reported endogenous levels [25,27]. The LLOQ reported for NE by [25] (0.3 ng/mL) was lower than the LLOQ reported here (3.2 ng/mL). These studies used an API 4000 QTrap mass spectrometer in positive mode (as used here), but a different extraction procedure (single extraction with *n*-heptane/ ethyl acetate (1/1, v/v)), solvent composition for the LC (ACN and water both with 0.1% formic acid) and MRM transition (365/273). On the other hand, the method reported by Balvers et al. [27], which uses a similar sample preparation method (ACN for extraction followed by a cleanup using SPE), but a different mass spectrometer (Thermo Finnigan TSQ Quantum triple quadrupole), distinct solvent composition for the LC (methanol and water containing 13 mM ammonium acetate and 0.1% formic acid) and different MRM transitions (382 $[M+NH_4]^+$/91) shows a higher LLOQ (647 pg on column) as compared to ours (32 pg on column).

The basal levels of PGE_2 and THB B_2 reported by Shinde et al. [24] are below our LOD. It is known that prostanoid levels can increase in response to inflammation and pathophysiological conditions [48] and that the method can measure increased levels in plasma of PGE_2 and THB B_2. In 4 of the 32 samples analyzed, we observed THB B_2 at the LOD. The ALDO levels reported here with our method are higher that the levels reported in the literature, which compare radioimmunoassay (RIA) with LC-MS/MS (negative mode) [29,30]. Both literature sources showed good correlations between the two methods of analysis (higher variation with RIA), however, only one of them provided information about the sample collection (samples were collected at midmorning from normotensive patients [29]). The time at which the blood was collected and the condition of the subjects (fasting or not) are known to be critical in the analysis of steroids. The levels of aldosterone reported in the present study are in agreement with those reported by Hollenberg et al. [49], who used RIA for the analysis of plasma samples. In that study, the samples were collected in the morning under fasting conditions that are similar to those used in our study.

Different studies have reported levels of THDOC and THP in human and rodent plasma using gas chromatography chemical ionization mass spectrometry (GC-MS) or radioimmunassays [50–55]. Using our LC-MS/MS, we were unable to detect these

analytes. In the case of THP, the LOD/LLOQ reported is the only reference to our knowledge that also includes analytical information about the method (ca. 1 pg/ 10 pg on column [50]) is lower that our LOD/ LLOQ (160/ 400 pg on column (see Table 2)). Accordingly, only very low levels of THP were reported for plasma (77±20 pg/mL or 0.2+0.1 pmol/mL) in this study, leading us to question the physiological relevance of these amounts. With respect to the analysis of THDOC, most articles refer to Romeo et al. [56,57], in which a full method description is missing. Nevertheless, our LOD for THDOC (2.5 ng on column) was much higher than the one reported for THP (see Table 2). Furthermore, our method was not able to detect endogenous concentrations of A-GABA, A-GLY, A-SER, ANDRO, PGE_2EA and NADA in human blood plasma. In agreement with our findings, two studies have tried to measure NADA and A-GLY in human plasma without success [25,27]. So far, A-GABA and A-SER have only been identified in the mouse brain [58].

5. Conclusions

In this study we validated an analytical method that enables researchers to simultaneously measure bioactive lipids from different metabolite networks. The present method is sensitive, accurate and precise. It allows the quantification of selected prostanoids, ECs, *N*-acylethanolamines and steroids in human plasma. This analytical LC-MS/MS method enables the analysis of co-variations of these metabolites in human plasma under pathophysiological conditions.

Acknowledgements

This work was financially supported by the NCCR TransCure and the SNF grant 141174 (JG). We would like to acknowledge Stefan König and Jelena Simovic Rota for valuable technical support and Sarah Crocket for English editing.

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Table 1

MRMs and chromatographic conditions for the analytes and IS evaluated in human plasma.

Analyte	IS used	Retention time (min)	Q/q	Precursor ion (*m/z*)	Product ion (*m/z*)	Declustering potential (eV)	Collition energy (eV)	Observation
Analytes measured in negative mode								
AA	AA-d_8	8.57	Q	303	59	-80	-37	
			q	303	205	-80	-18	
PGE_2	PGE_2-d_4	5.37	Q	351	315	-70	-25	
			q	351	271	-70	-16	
THB B_2	PGE_2-d_4	5.25/5.47	Q	369	195	-62	-20	double peak
			q	369	169	-62	-23	
Analytes measured in positive mode								
AEA	AEA-d_4	8.02	Q	348	62	56	42	
			q	348	133	56	33	
2-AG	2-AG-d_5	8.11/8.22	Q	379	203	82	25	double peak
			q	379	287	51	18	
A-GABA	AEA-d_4	8.03	nd	390	86	68	45	
			nd	390	104	68	22	
A-GLY	AEA-d_4	7.93	nd	362	203	70	21	
			nd	362	269	70	18	
ALDO	COR-d_4	4.77	Q	361	109	80	48	
			q	361	97	80	52	
ANDRO	TEST-d_4	6.34	nd	291	135	76	30	fronting
			nd	291	147	76	32	
A-SER	AEA-d_4	7.78	nd	392	106	63	22	
			nd	392	287	63	15	
COR	COR-d_4	4.89	Q	363	97	76	48	fronting
			q	363	121	76	38	
DHEA	TEST-d_4	5.61/5.76	Q	289	253	72	15	double peak
			q	289	213	72	26	
LEA	LEA-d_4	7.97	Q	324	62	74	36	
			q	324	109	74	32	
NADA	AEA-d_4	8.06	nd	440	137	75	34	
			nd	440	154	75	23	
NE	1/2-AG-d_5	8.42	Q	365	121	86	29	tailing
			q	365	133	86	40	
OEA	OEA-d_4	8.38	Q	326	62	72	36	
			q	326	309	72	21	
PEA	PEA-d_5	8.21	Q	300	62	78	36	
			q	300	283	78	19	
PGE2EA	AEA-d_4	5.02	nd	396	62	35	49	fronting
			nd	396	360	35	16	
PROG	PROG-d_9	6.25	Q	315	109	83	36	
			q	315	97	83	34	
SEA	PEA-d_5	8.81	Q	328	62	72	31	
			q	328	311	72	22	
TEST	TEST-d_4	5.61	Q	289	97	80	33	
			q	289	109	80	36	
THDOC	GANA	6.31	nd	335	135	80	30	
			nd	335	281	80	16	
THP	GANA	7.09	nd	319	301	70	13	
			nd	319	257	70	18	
VIR*	AEA-d_4	7.05	nd	348	62	62	41	
			nd	348	203	62	21	
IS measured in negative mode								
AA-d_8		8.53	IS	311	59	-90	-38	
PGE_2-d_4		5.36	IS	355	319	-64	-17	
IS measured in positive mode								
AEA-d_4		8.01	IS	352	66	60	35	
2-AG-d_5		8.09/8.20	IS	384	287	62	17	double peak
COR-d_4		4.88	IS	367	121	78	35	
GANA		7.54	IS	333	297	76	18	
LEA-d_4		7.96	IS	328	66	70	35	
OEA-d_4		8.38	IS	330	66	72	32	
PEA-d_5		8.21	IS	305	62	70	40	
PROG-d_9		6.21	IS	324	100	83	37	
TEST-d_4		5.6	IS	293	98	80	32	

*VIR could only been see the standards. Therefore, it was not further asseed.
nd: not determine
Q: MRM transition used as quantifier
q: MRM transition used as quanlifier
IS: internal standard

Table 2

LODs and LLOQs of all MRM transitions of the analytes evaluated.

Analyte	Q/q	Precursor ion (*m/z*)	Product ion (*m/z*)	LOD (ng/mL)	LOD (pg on column)	LOD (fmol on column)	LOQ (ng/mL)	LOQ (pg on column)	LOQ (fmol on column)
AA	Q	303	59	12.3[i]	123.0	404.6	192	1920.0	6315.8
	q	303	205	12.3i	123.0	404.6	192	1920.0	6315.8
AEA	Q	348	62	0.04[i]	0.4	1.2	0.1	1.0	2.9
	q	348	133	0.1[i]	1.0	2.9	0.6	6.4	18.4
2-AG	Q	379	203	0.6[i]	6.0	15.9	3.2	32.0	84.7
	q	379	287	0.3[i]	3.0	7.9	3.2	32.0	84.7
A-GABA	nd	390	86	0.2	2.0	5.1	<0.5	5.0	12.9
	nd	390	104	0.2	2.0	5.1	<0.5	5.0	12.9
A-GLY	nd	362	203	3.2	32.0	88.6	8	80.0	221.6
	nd	362	269	3.2	32.0	88.6	8	80.0	221.6
ALDO	Q	361	109	0.5[i]	5.0	13.9	3.2	32.0	88.9
	q	361	97	0.5i	5.0	13.9	3.2	32.0	110.3
ANDRO	nd	291	135	40	400.0	1379.3	100	1000.0	3448.3
	nd	291	147	100	1000.0	3448.3	250	2500.0	8620.7
A-SER	nd	392	106	3.2	32.0	81.8	8	80.0	204.6
	nd	392	287	3.2	32.0	81.8	8	80.0	204.6
COR	Q	363	97	0.4[i]	4.0	11.0	6.4	64.0	176.8
	q	363	121	0.4i	4.0	11.0	6.4	64.0	176.8
DHEA	Q	289	253	2.6[i]	25.6	88.9	2.6	25.6	88.9
	q	289	213	2.6[i]	25.6	88.9	2.6	25.6	88.9
LEA	Q	324	62	0.04[i]	0.4	1.2	0.1	1.0	3.1
	q	324	109	0.6[i]	6.0	18.6	1.6	16.0	49.5
NADA	nd	440	137	4	40.0	91.1	10	100.0	227.8
	nd	440	154	4	40.0	91.1	10	100.0	227.8
NE	Q	365	121	1.3[i]	12.8	35.2	3.2	32.0	87.9
	q	365	133	3.2[i]	32.0	87.9	3.2	32.0	87.9
OEA	Q	326	62	0.08[i]	0.8	2.5	0.51	5.1	15.7
	q	326	309	0.2[i]	2.0	6.2	1.28	12.8	39.4
PEA	Q	300	62	0.08[i]	0.8	2.7	3.2	32.0	107.0
	q	300	283	0.51[i]	5.1	17.1	8	80.0	267.6
PGE_2	Q	351	315	0.2[i]	2.0	5.7	0.51[i]	5.1	14.5
	q	351	271	0.2[i]	2.0	5.7	0.51[i]	5.1	14.5
PGE2EA	nd	396	62	32	320.0	810.1	80	800.0	2025.3
	nd	396	360	200	2000.0	5063.3	500	5000.0	12658.2
PROG	Q	315	109	0.04[i]	0.4	1.3	0.1	1.0	3.2
	q	315	97	0.04[i]	0.4	1.3	0.1	1.0	3.2
SEA	Q	328	62	0.08[i]	0.8	2.4	8	80.0	244.6
	q	328	311	0.51[i]	5.1	15.6	8	80.0	244.6
TEST	Q	289	97	0.04[i]	0.4	1.4	0.1	1.0	3.5
	q	289	109	0.04[i]	0.4	1.4	0.1	1.0	3.5
THDOC	nd	335	135	250	2500.0	7485.0	500	5000.0	14970.1
	nd	335	281	250	2500.0	7485.0	500	5000.0	14970.1
THP	nd	319	301	16[i]	160.0	503.1	40[i]	400.0	1257.9
	nd	319	257	16[i]	160.0	503.1	40[i]	400.0	1257.9
THB B_2	Q	369	195	0.51	5.1	13.8	3.2	32.0	86.5
	q	369	169	0.51	5.1	13.8	3.2	32.0	86.5

[i] Intrumental limit of detection

Table 3

Dynamic range, linearity and accuracy of the MRM transitions selected for the quantification of ECs, *N*-acylethanolamines, prostanoids and steroids found in human plasma.

	Q						Accuracy plasma	
Analyte	**Precursor ion (*m/z*)**	**Product ion (*m/z*)**	**Dynamic range mesured (ng/mL)**	**R^2(±SD, n=3)**	**Slope**	**Intercept**	**Amount spiked (ng/mL)**	**(%, n=6)**
AA	303	59	192-15000	0.998±0.002	0.0007±0.00003	0.8846±0.7670	3000	136±24
							7500	130±13
							15000	115±8
AEA	348	62	0.1-50	0.993±0.006	0.0596±0.0035	0.0131±0.0469	1.6	132±48
							4	110±21
							10	113±14
2-AG	379	203	1.28-100	0.984±0.016	0.0108±0.0010	-0.024±0.065	20	70±49
							50	106±20
							100	105±20
ALDO	361	109	3.2-100	0.999±0.001	0.0019±0.0001	0.0015±0.0011	3.2	111±43
							8	113±20
							20	103±13
COR	363	97	6.4-1000	0.996±0.002	0.0015±0.00003	0.0182±0.0168	250	113±10
							500	106±9
							1000	106±4
DHEA	289	253	2.6-500	0.994±0.005	0.0003±0.00001	0.0033±0.0014	40	88±52
							100	83±51
							250	79±49
LEA	324	62	0.1-50	0.992±0.005	0.0160±0.0004	0.0136±0.0045	4	121±15
							10	108±8
							25	114±11
NE	365	121	3.2-100	0.991±0.012	0.0122±0.0005	0.0212±0.0106	3.2	114±35
							8	105±25
							20	112±17
OEA	326	62	0.51-100	0.998±0.002	0.0735±0.0053	.-0.0034±0.0232	8	103±18
							20	103±9
							50	104±8
PEA	300	62	3.2-100	0.995±0.003	0.0080±0.0005	0.0060±0.0077	8	96±24
							20	100±13
							50	105±16
PGE_2	351	315	3.2-100	0.993±0.009	0.0369±0.0049	0.0704±0.1172	20	103±22
							50	102±17
							100	96±16
PROG	315	109	0.1-50	0.999±0.001	0.0868±0.0039	0.0264±0.0056	0.64	114±16
							1.6	108±11
							4	108±14
SEA	328	62	8-100	0.995±0.004	0.0070±0.0011	0.0073±0.0113	8	91±25
							20	102±29
							50	93±21
TEST	289	97	0.1-50	0.999±0.001	0.0818±0.0052	0.0245±0.0068	10	102±9
							25	101±9
							50	97±8
THB B_2	369	195	0.51-100	0.998±0.003	0.0437±0.0035	0.0236±0.0102	1.28	105±20
							8	130±18
							20	102±14

Table 4

Analysis of quality control samples (QC): Intra- and inter-day precision and recovery analyses.

			QC_0 (n=6)				QC_4 (n=6) Inter-day			QC_7 (n=6) Inter-day		
			Intra-day		Inter-day							
Analyte	**Precursor ion (*m/z*)**	**Product ion (*m/z*)**	**Calculated conc. (pmol/mL)**	**CV (%)**	**Calculated conc. (pmol/mL)**	**CV (%)**	**Amount spiked (ng/mL)**	**Recovery (%)**	**CV (%)**	**Amount spiked (ng/mL)**	**Recovery (%)**	**CV (%)**
AA	303	59	5524.3±198.8	3.9	4882.8±755.7	15.5	192	116	19.3	3000	129	16.2
AEA	348	62	1.4±0.3	19.6	1.4±0.2	16.1	0.64	88	25.8	10	128	11.1
2-AG	379	203	42.8±2.8	6.1	72.2±9.8	13.6	1.28	[a]	16.2	20	118	15.1
ALDO	361	109	5.4±0.3	5.0	3.4±1.0	29.3	1.28	96	4.6	20	128	8.3
COR	363	97	126.6±4.7	3.5	117.0±9.9	8.4	6.4	98	10.9	100	134	10.3
DHEA	289	253	13.2±1.8	10.4	15.8±4.1	25.7	6.4	104	24.1	100	115	9.5
LEA	324	62	3.3±0.4	10.6	3.9±0.4	9.3	0.64	95	14.0	10	120	9.4
NE	365	121	5.6±0.5	8.5	10.1±3.8	37.5	1.28	73	41.2	20	108	23.9
OEA	326	62	7.6±0.3	4.5	7.1±0.8	11.3	1.28	113	6.9	20	127	15.1
PEA	300	62	19.6±1.6	8.2	22.3±3.9	17.7	1.28	[a]	20.5	20	109	16.3
PGE_2	351	315	*	*	*	*	1.28	*	*	20	*	13.3
PROG	315	109	0.6±0.05	3.3	0.5±0.1	12.5	0.64	134	9.2	10	129	11.5
SEA	328	62	15.9±1.1	11.2	19.8±5.1	26.0	1.28	104	18.3	20	104	22.0
TEST	289	97	4.3±0.2	3.5	8.5±0.8	9.4	0.64	79	7.5	10	119	12.3
THB B_2	369	195	0.3±0.1	15.1	<lod	-	1.28	77	44.0	20	94	13.3

* Peaks coming at the same retention time (signal overlapping)

[a] Not possible to obatain the recovery (amount spiked was too little)

Table 5

Dynamic range, linearity and accuracy of the MRM transitions selected for the quantification of ECs, *N*-acylethanolamines, prostanoids and steroids found in human plasma.

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Analytical dinamic range (ng/mL)	Analytical amount found in plasma; n=32 (ng/mL)	Concentration found in plasma of healthy subjects; n=32 (pmol/mL)	Plasma concentration reported in literature values from healthly subjects (pmol/mL)
AA	303	59	192-15000	6063±2692	5063±2606	3759.9±1539.5 (n=6, M) [24]
AEA	348	62	0.1-50	2.6±0.7	1.7±0.4	0.1-4.6 ** [40]; 0.3-1.5 (n=5, MF) [25]; 0.7±0.02 (n=ns, M) [26]; 0.7±0.3 (n=23, F) [27]; 0.72±0.22 (n= ns, ns) [28]
2-AG	379	203	1.28-100	31.2±10.0	16.5±5.3	0.6-18.7** [40]; 5.7-10.6 (n=5, MF) [25]; 18.8+32.8 (n=ns, M) [26]; 18.8±32.8 (n=23,F) [27]
ALDO	361	109	3.2-100	14.8±2.6	8.2±1.4	7.2±0.7 (n=58, MF, RIA) [49]; <0.1-0.6 (n=97, MF) [29]; 0.1-1.0
COR	363	97	6.4-1000	640±184	354±102	100-790 (n=9, MF) [10]; 101.2-713.6 (2.5-97.5% of n=66, M (>16 years old)) [31]
DHEA	289	253	2.56-500	46.9±11.0	32.6±7.6	5-45 (n=12, M; GCMS) [42]
LEA	324	62	0.1-50	6.7±1.9	4.1±1.2	nr
OEA	326	62	0.51-100	16.0±4.0	9.9±2.4	4.9±0.4 (n=ns ,M) [26]; 4.2±1.3 (n=23, F) [27]
PEA	300	62	3.2-100	34.4±7.7	23.0±5.2	5.2+0.5 (n=ns ,M) [26]; 4.6±1.2 (n=23, F) [27]
PGE_2	351	315	3.2-100	<lod	-	0.08±0.04 (n=6, M) [24]
PROG	315	109	0.1-50	0.5±0.2	0.3±0.1	0.32-42.45 (n=20 F, full cycle CIA) [43]; 4-65 (n=22, MF, GCMS) [44]; 0.1-0.7 (2.5-97.5% of n=66, M (>16 years old) [31]
SEA	328	62	8-100	21.5±9.7	13.1±5.9	1.6±0.6 (n=23, F) [27]
TEST	289	97	0.1-50	19.7±4.8	13.7±3.4	8-90 (n=12 M; GCMS) [42]
THB B_2	369	195	0.51-100	0.1±0.1**	0.4±0.4**	0.48±0.18 (n=6, M) [24]

M= male; F= female; MF= male and female; pmF=postmenopausal women

*Only a few samples contain THB B_2 slightly above the LOD

**n=4; <LOQ

ns= not specified

CIA= chemiluminescent immunoassay

RIA= radioimmunoassay

GCMS= gas chromatography mass spectromety

nr= not reported

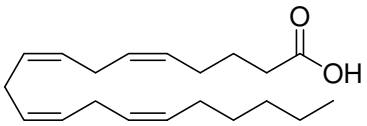
Supplementary Table 1

Instrumental LOD of the MRM transitions of the analytes that were not found in human plasma in this study.

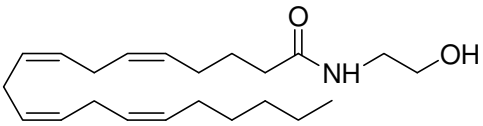
Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	LOD (ng/mL)	LOD (pg on column)	LOD (fmol on column)
A-GABA	390	86	0.08	0.8	2.1
	390	104	0.08	0.8	2.1
A-GLY	362	203	3.2	32.0	88.6
	362	269	3.2	32.0	88.6
ANDRO	291	135	6.4	64.0	220.7
	291	147	6.4	64.0	220.7
A-SER	392	106	1.28	12.8	32.7
	392	287	1.28	12.8	32.7
NADA	440	137	1.28	12.8	29.2
	440	154	1.28	12.8	29.2
PGE2EA	396	62	20	200.0	506.3
	396	360	20	200.0	506.3
THDOC	335	135	40	400.0	1197.6
	335	281	40	400.0	1197.6
THP	319	301	16	64.0	201.3
	319	257	16	64.0	201.3
VIR	348	62	3.2	32.0	86.5
	348	203	8	80.0	216.2

Fig. 1. Chemical structures of the analytes measured in human plasma with LC-MS/MS. A: Endocannabinoids, *N*-acylethanolamines, prostanoids. B: Steroids

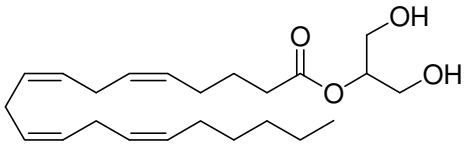
A



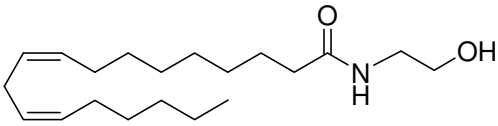
Arachidonic acid (AA)
$C_{20}H_{32}O_2$
Exact Mass: 304.2



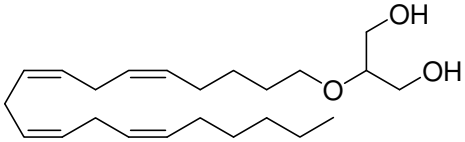
Anandamide (AEA)
$C_{22}H_{37}NO_2$
Exact Mass: 347.3



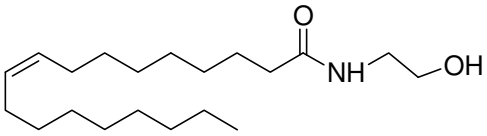
2-Arachidonoyl glycerol (2-AG)
$C_{23}H_{38}O_4$
Exact Mass: 378.3



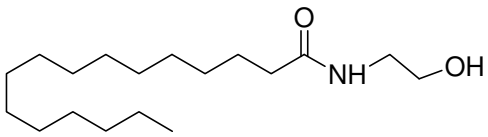
Linoleoyl ethanolamide (LEA)
$C_{20}H_{37}NO_2$
Exact Mass: 323.3



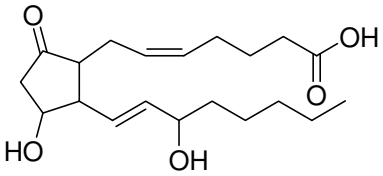
Noladin ether (NE)
$C_{23}H_{40}O_3$
Exact Mass: 364.3



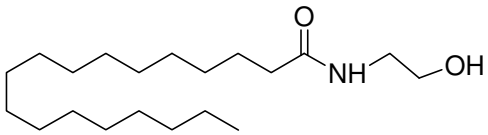
Oleoyl ethanlonamide (OEA)
$C_{20}H_{39}NO_2$
Exact Mass: 325.3



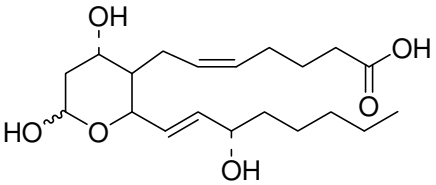
Palmitoylethanolamide (PEA)
$C_{18}H_{37}NO_2$
Exact Mass: 299.3



Prostaglandin E_2 (PRE_2)
$C_{20}H_{32}O_5$
Exact Mass: 352.2

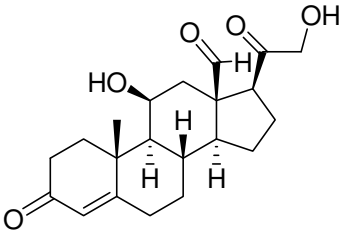


Stearoyl ethanolamide (SEA)
$C_{20}H_{41}NO_2$
Exact Mass: 327.3

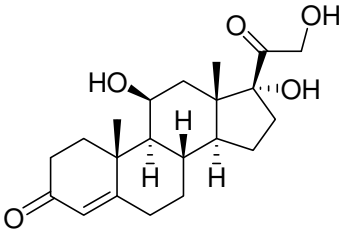


Thromboxane B_2 (THB B_2)
$C_{20}H_{34}O_6$
Exact Mass: 370.2

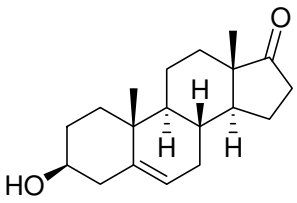
B



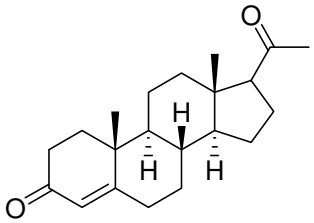
Aldosterone (ALDO)
$C_{21}H_{28}O_5$
Exact Mass: 360.2



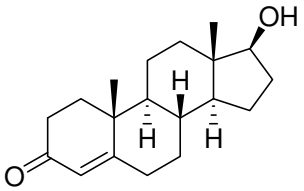
Cortisol (COR)
$C_{21}H_{30}O_5$
Exact Mass: 362.2



Dehydroepiandrosterone (DHEA)
$C_{19}H_{28}O_2$
Exact Mass: 288.2



Progesterone (PROG)
$C_{21}H_{30}O_2$
Exact Mass: 314.2



Testosterone (TEST)
$C_{19}H_{28}O_2$
Exact Mass: 288.2

Fig. 2. Chromatograms of the analytes measured in positive mode. A: Chromatogram showing MRM transitions of analytes and internal standards (IS) of “only standards”. B and C: Chromatograms of individual analytes. First row: analytes of “only standards”, second row: IS of “only standards”, third row: endogenous levels of analytes (one representative example of n=32) found in plasma and fourth row: IS in plasma.

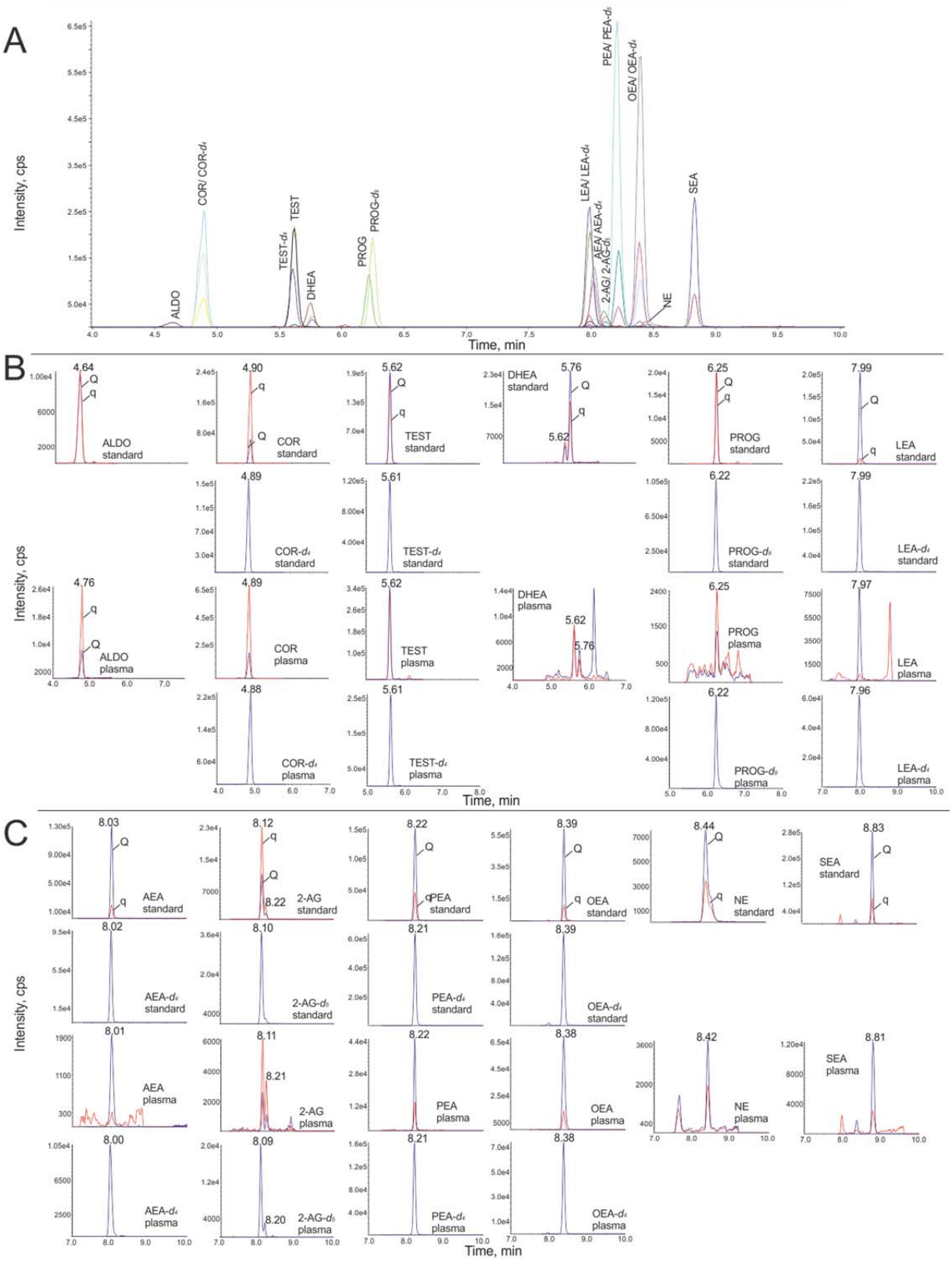
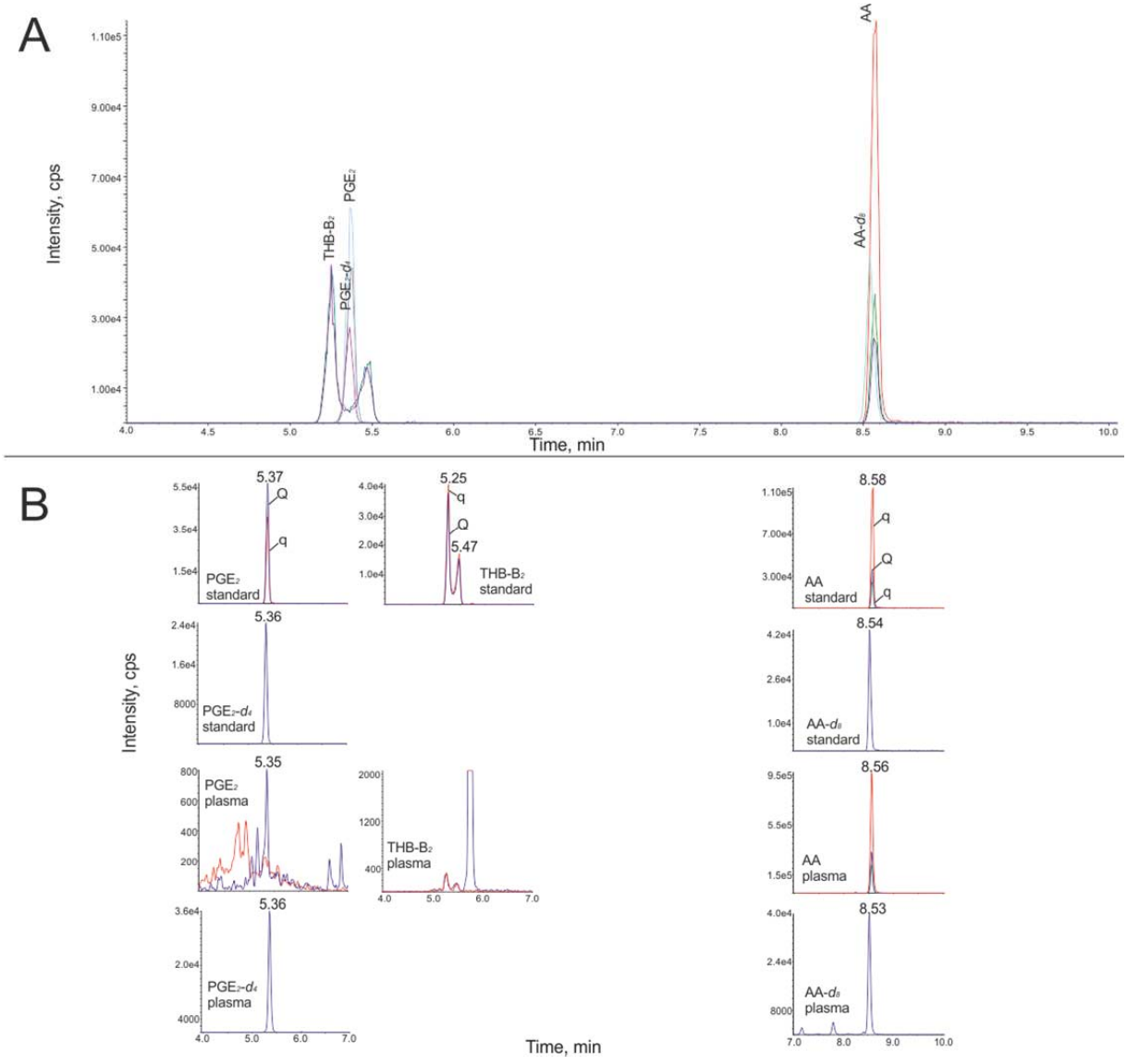
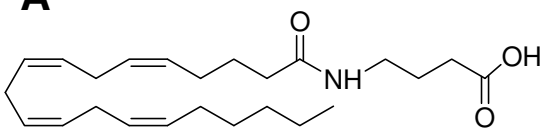

A
Intensity, cps
Time, min
ALDO
COR/ COR-d4
TEST-d4
TEST
DHEA
PROG
PROG-d9
LEA/ LEA-d4
AEA/ AEA-d8
2-AG/ 2-AG-d5
PEA/ PEA-d4
OEA/ OEA-d4
NE
SEA
B
Intensity, cps
Time, min
ALDO standard
COR standard
TEST standard
DHEA standard
PROG standard
LEA standard
COR-d4 standard
TEST-d4 standard
PROG-d9 standard
LEA-d4 standard
ALDO plasma
COR plasma
TEST plasma
DHEA plasma
PROG plasma
LEA plasma
COR-d4 plasma
TEST-d4 plasma
PROG-d9 plasma
LEA-d4 plasma
C
Intensity, cps
Time, min
AEA standard
2-AG standard
PEA standard
OEA standard
NE standard
SEA standard
AEA-d4 standard
2-AG-d5 standard
PEA-d4 standard
OEA-d4 standard
AEA plasma
2-AG plasma
PEA plasma
OEA plasma
NE plasma
SEA plasma
AEA-d4 plasma
2-AG-d5 plasma
PEA-d4 plasma
OEA-d4 plasma

Figure 3: Chromatograms of the **analytes validated** with the presented method run in positive mode. A: Chromatogram showing MRM transitions of analytes and internal standards (IS) of “only standards”. B: Individual chromatograms of analytes. First raw: analytes of “only standards”, second raw: IS of “only standards”, third raw: endogenous levels of analytes (e.g. one of n=32) find “in plasma” and fourth raw: IS recovered from analysis “in plasma”.

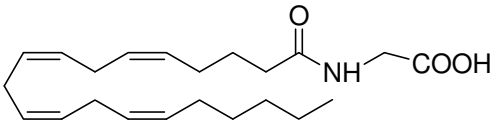


Supplementary Figure 1: Chemical structures of the analytes investigated that were not detected in human plasma (Not validated). A: Endocannabinoid-like lipids, B: Steroids

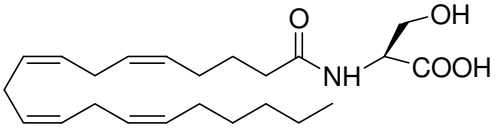
A



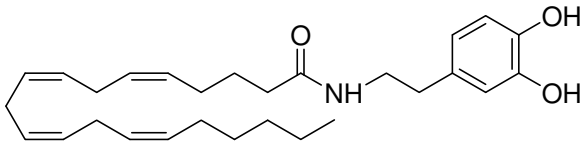
***N*-arachidonyl-GABA** (A-GABA)
$C_{24}H_{39}NO_3$
Exact Mass: 389.3



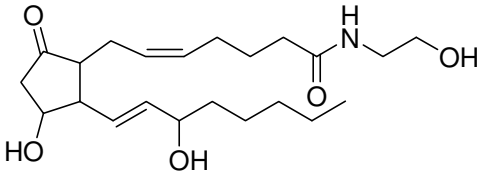
***N*-arachidonoyl glycine** (A-GLY)
$C_{22}H_{35}NO_3$
Exact Mass: 361.3



***N*-arachidonoyl-L-Serine** (A-SER)
$C_{23}H_{37}NO_4$
Exact Mass: 391.3

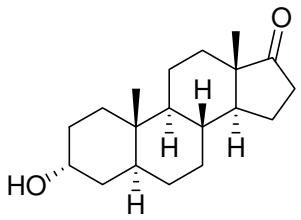


***N*-arachidonoyl dopamide** (NADA)
$C_{28}H_{41}NO_3$
Exact Mass: 439.3

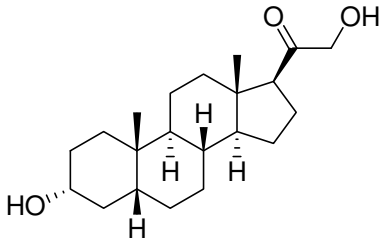


Prostaglandin E_2 ethanolamide (PGE_2EA)
$C_{22}H_{37}NO_5$
Exact Mass: 395.3

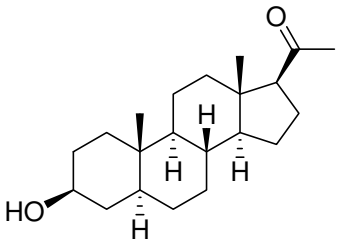
B



Androsterone (ANDRO)
$C_{19}H_{30}O_2$
Exact Mass: 290.2



Tetrahydrodeoxycorticosterone (THDOC)
$C_{21}H_{34}O_3$
Exact Mass: 334.3



Allopregnanolone (THP)
$C_{21}H_{34}O_2$
Exact Mass: 318.3

Supplementary Figure 2: Chromatograms of the analytes investigated that were not detected in human plasma. The top panel shows a chromatogram of all the analytes investigated. The bottom panel shows individual chromatograms of the two MRM transitions of the analytes that were not detected in human plasma and not further validated.