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Simultaneous UPLC–MS/MS quantification of the endocannabinoids 2-arachidonoyl glycerol (2AG), 1-arachidonoyl glycerol (1AG), and anandamide in human plasma: Minimization of matrix-effects, 2AG/1AG isomerization and degradation by toluene solvent extraction☆

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ABSTRACT

Analysis of the endocannabinoid (EC) system's key molecules 2-arachidonoyl glycerol (2AG) and arachidonoyl ethanolamide (anandamide, AEA) is challenging due to several peculiarities. 2AG isomerizes spontaneously to its biologically inactive analogue 1-arachidonoyl glycerol (1AG) by acyl migration and it is only chromatographically distinguishable from 1AG. Matrix-effects caused primarily by co-extracted phospholipids may further compromise analysis. In addition, 2AG and 1AG are unstable under certain conditions like solvent evaporation or reconstitution of dried extracts. We examined effects of different organic solvents and their mixtures, such as toluene, ethyl acetate, and chloroform-methanol, on 2AG/1AG isomerisation, 2AG/1AG stability, and matrix-effects in the UPLC–MS/MS analysis of 2AG and AEA in human plasma. Toluene prevented, both, 2AG isomerisation to 1AG and degradation of 2AG/1AG during evaporation. Toluene extracts contain only 2% of matrix-effect-causing plasma phospholipids compared to extracts from the traditionally used solvent mixture chloroform–methanol. Toluene and all other tested organic solvents provide comparable 2AG and AEA extraction yields (60–80%). Based on these favourable toluene properties, we developed and validated a UPLC–MS/MS method with positive electrospray ionization (ESI+) that allows for simultaneous accurate and precise measurement of 2AG and AEA in human plasma. The UPLC–MS/MS method was cross-validated with a previously described fully-validated GC–MS/MS method for AEA in human plasma. A close correlation ($r^2 = 0.821$) was observed between the results obtained from UPLC–MS/MS (y) and GC–MS/MS (x) methods ($y = 0.01 + 0.85x$). The UPLC–MS/MS method is suitable for routine measurement of 2AG and AEA in human plasma samples (1 mL) in clinical settings as shown by quality control plasma samples processed over a period of 100 days. The UPLC–MS/MS method was further extended to human urine. In urine, AEA was not detectable and 2AG was detected in only 3 out of 19 samples from healthy subjects at 160, 180 and 212 pM corresponding to 12.3, 14.5 and 9.9 pmol/mmol creatinine, respectively.

1. Introduction

2-Arachidonoyl glycerol (2AG) and arachidonoyl ethanolamide (see Fig. 1), better known as anandamide (AEA), are endogenous lipid mediators that bind to cannabinoid (CB) receptors [1]. Therefore, 2AG and AEA are also named endocannabinoids (EC). AEA is fairly stable and an isomerisation to arachidonoyl ethanolamine ester (virodhamine) only occurs very rarely under unusual conditions (see Fig. 1). By contrast, it is well known that under certain common experimental conditions 2AG spontaneously isomerizes to 1-arachidonoyl glycerol (1AG) (see Fig. 1), which is biologically inactive [2]. This phenomenon is also known as acyl migration. Elevated temperatures, presence of serum albumin, and high pH values have been reported to accelerate 2AG/1AG acyl migration [3]. It is still unclear whether 1AG found in human plasma and in other biological samples is an endogenously produced primary arachidonic acid metabolite, or whether it solely originates from 2AG via acyl migration. Because of the lack of detailed knowledge

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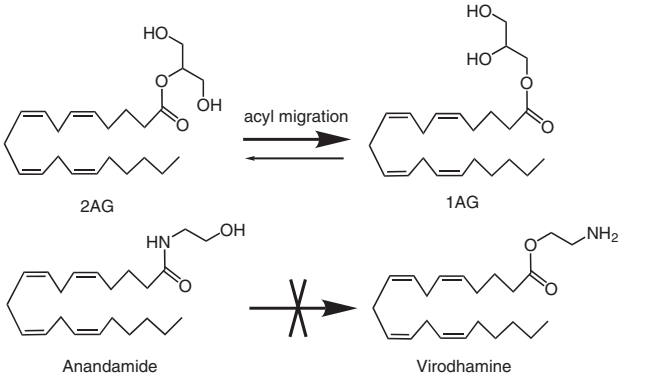

Fig. 1. Chemical structures of 2AG, 1AG, anandamide (AEA) and virodhamine. Isomerisation of 2AG to 1AG can be observed under standard conditions, whereas a conversion of anandamide to its isomer virodhamine has not been reported under standard conditions thus far.

about the chemistry of the 2AG/1AG system, simultaneous analysis of 2AG and 1AG in biological samples or specific analysis of 2AG without changing a putative endogenous 2AG/1AG equilibrium state is challenging. A commonly used approach is to dispense with a chromatographic separation of 2AG from 1AG and to assign the acquired single peak entirely to 2AG [4]. Another proposed and commonly applied approach is based on the chromatographic separation of 2AG and 1AG, the summation of the acquired individual peak areas, and the assumption that 1AG originates exclusively from 2AG [5].

At present, LC–MS/MS with positive electrospray ionization (ESI+) is the most frequently used technique for the quantitative determination of 2AG and AEA in biological samples [4,6–14]. For the measurement of AEA in human plasma a GC–MS/MS method has also been reported [15]. In general, LC–MS/MS-based methods are associated with problems originating from the biological matrix [16–18]. Phospholipids present in human plasma and other biological samples have been recognized as a major contributor to matrix-effects in LC–MS/MS methods employed for numerous endogenous substances [19,20].

The aim of the present work was to develop and validate a UPLC–MS/MS method for the simultaneous accurate and precise measurement of 2AG and AEA in human plasma. We investigated by LC–MS/MS the effects of 2AG solvent extraction from human plasma by means of various organic solvents including toluene and those used in the traditional extraction methods reported by Folch or by Bligh and Dyer [21,22]. We also examined the solvent extracts for 2AG/1AG isomerisation, 2AG/1AG stability, and content of matrix-effect-causing phospholipids. We found that toluene is best suited for the specific quantitation of 2AG and AEA in peripheral human plasma in clinical studies. The toluene-based UPLC–MS/MS method was cross-validated for AEA in 250 human plasma samples by a previously reported fully-validated GC–MS/MS method which also applies solvent extraction by toluene [15].

2. Experimental

2.1. Chemicals and materials

In the present work chemicals are reported with their trivial names. For the IUPAC nomenclature of 2AG, 1AG, AEA and of their stable isotope-labeled analogues see Table 1. All unlabeled and deuterium-labeled EC were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Unlabeled 2AG (d_0-2AG), [$^2H_5$]-2AG (d_5-2AG) and [$^2H_8$]-2AG (d_8-2AG) were declared to have a chemical purity of ≥95%, and to contain 10% of unlabeled and labeled 1AG, respectively. Unlabeled AEA (d_0-AEA), [$^2H_4$]-AEA (d_4-AEA) and [$^2H_8$]-AEA (d_8-AEA) were declared to have a chemical purity of ≥98%. The declared isotopic purity was ≥99% at ^2H for d_4-AEA, d_8-AEA, d_5-2AG and d_8-2AG. All analytes were supplied as solutions in acetonitrile (2AG) or ethanol (AEA); dilutions were prepared without solvent evaporation. HPLC-grade organic solvents and LC–MS grade methanol were from Mallinckrodt Baker (Griesheim, Germany). Distilled water was delivered by DeltaSelect (Pfullingen, Germany). Ammonium acetate of LC–MS grade was from Sigma–Aldrich (Munich, Germany). All glass vials used came from Macherey-Nagel (Düren, Germany). Polypropylene tubes and vials for blood sampling were manufactured by Sarstedt (Nümbrecht, Germany).

Table 1
IUPAC nomenclature of 2AG, 1AG and AEA standards used.

Unlabeled 2AG (d_0-2AG)	1,3-dihydroxypropan-2-yl (5*Z*,8*Z*,11*Z*,14*Z*)-eicosatetraenoate
[$^2H_5$]-2AG (d_5-2AG)	1,3-[1,1,2,3,3-$^2H_5$]-dihydroxypropan-2-yl (5*Z*,8*Z*,11*Z*,14*Z*)-icosatetraenoate
[$^2H_8$]-2AG (d_8-2AG)	1,3-dihydroxypropan-2-yl (5*Z*,8*Z*,11*Z*,14*Z*)-[5,6,8,9,11,12,14,15-$^2H_8$]-eicosatetraenoate
Unlabeled 1AG (d_0-1AG)	2,3-dihydroxypropan-1-yl (5*Z*,8*Z*,11*Z*,14*Z*)-eicosatetraenoate
[$^2H_5$]-1AG (d_5-1AG)	2,3-[1,1,2,3,3-$^2H_5$]-dihydroxypropan-1-yl (5*Z*,8*Z*,11*Z*,14*Z*)-icosatetraenoate
Unlabeled AEA (d_0-AEA)	(5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(2-hydroxyethyl)eicosa-5,8,11,14-tetraenamide
[$^2H_4$]-AEA (d_4-AEA)	(5*Z*,8*Z*,11*Z*,14*Z*)-*N*-([1,1,2,2-$^2H_4$]-2-hydroxyethyl)eicosa-5,8,11,14-tetraenamide
[$^2H_8$]-AEA (d_8-AEA)	(5*Z*,8*Z*,11*Z*,14*Z*-[5,6,8,9,11,12,14,15-$^2H_8$])-*N*-(2-hydroxyethyl)-eicosa-5,8,11,14-tetraenamide

2.2. Instrumentation

Analyses were performed on a Waters ACQUITY UPLC–MS/MS system consisting of a solvent delivery device, an autosampler, a column thermostat and the tandem quadrupole mass spectrometer XEVO TQ MS (Waters, Milford, MA, USA). Mobile phase A was water which was daily prepared by means of a Milli-Q Synthesis A10 System (Millipore, Billerica, MA, USA) and contained 2 mM ammonium acetate. Mobile phase B was methanol and contained 2 mM ammonium acetate. The following gradient was used: 0.0–0.5 min 75% B, 0.5–5.0 min to 79% B, 5.0–5.5 min to 90% B, and 5.5–6.5 min 75% B. The flow rate was kept constant at 0.5 mL/min. Separation of analytes was carried out on a Waters ACQUITY BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm particle size) thermostated at 60 °C.

Electrospray ionization in the positive (ESI+) and negative (ESI−) mode was used with nitrogen (600 °C, flow rate of 1100 L/h) as the desolvation gas. The capillary voltage was set to 0.8 kV, the ion source was kept at 150 °C. Argon served as the collision gas (0.13 mL/min, 1.8×10^{-3} mbar). Quantitative measurements were performed in the selected-reaction monitoring (SRM) mode. Parent ions and product ions used are summarized in Table 2.

2.3. Validation of the method

2.3.1. Linearity and standardization

The analytes concentrations reported in this section are approximate concentrations as declared by the supplier for the stock solutions of the analytes. A mixture containing d_5-2AG (53.3 pg/μL) and d_4-AEA (50 pg/μL) was prepared. To varying volumes (2, 5, 10, 20, 50 μL) of water–methanol (1:3, v/v) mixtures, a fixed volume (10 μL) of a stock solution containing unlabeled d_0-AEA and d_0-2AG (each 1000 pg/μL) was added. Each mixture was filled up to a total volume of 500 μL with water–methanol (1:3, v/v), and 25-μL aliquots of these solutions were analyzed by UPLC–MS/MS in the SRM mode. All samples were prepared and analyzed in duplicate.

Table 2
Mass transitions used in quantification of 2AG, 1AG and AEA by UPLC–MS/MS in the positive ESI mode.

Analyte	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Cone voltage (V)	Collision energy (eV)
d_0-2AG/d_0-1AG	379.4 $[M+H]^+$	287.3	25	15
d_5-2AG/d_5-1AG	384.4 $[M+H]^+$	287.3	25	15
d_8-2AG/d_8-1AG	387.4 $[M+H]^+$	294.3	25	15
d_0-AEA	348.4 $[M+H]^+$	62	22	14
d_4-AEA	352.4 $[M+H]^+$	66	22	14
d_8-AEA	356.4 $[M+H]^+$	62	22	14

Seven serial 1:5 (v/v)-dilutions of a mixture containing 100 pg/μL d_0-2AG and 200 pg/μL d_0-AEA in water–methanol (1:3, v/v) were prepared, with the lowest concentrations being 1.25 fg/μL and 2.5 fg/μL, respectively. Aliquots (20 μL) of these samples were analyzed by UPLC–MS/MS as described above and the limit of detection (LOD) of the method was determined. LOD was defined as the injected amount producing a peak with a signal-to-noise (peak-to-peak) ratio of 3:1.

2.3.2. Studies on solvent extraction

Aliquots (250 μL) from pooled human plasma (placed on ice) were spiked with a 9.7-μL aliquot of a standardized 100 pg/μL ethanolic solution of d_8-2AG and a 9-μL aliquot of a standardized 100 pg/μL ethanolic solution of d_8-AEA to reach added concentrations of 10 nM each. Six organic solvents commonly used for EC solvent extraction from human plasma were compared. Extraction was carried out with 750 μL aliquots of each of the following organic solvents and their mixtures: ethyl acetate, ethyl acetate–heptane (1:1, v/v), ethyl acetate–isohexane (9:1, v/v) and toluene; these organic solvents have been previously used for EC extraction [13,15,23,24]. In addition, we used the "Folch" and "Bligh & Dyer" method, i.e., methanol–chloroform mixtures, as originally described [21,22]. Extraction was performed in polypropylene plastic tubes using a Precellys 24 Dual Homogenizer (Bertin Technologies, Villeurbanne, France) for two 20-s lasting runs at 5000 rpm. Phases were separated by centrifugation (5 min, 4566 × *g*, 4 °C), except for "Folch" and "Bligh & Dyer" methods where saline was added for phase separation. The organic phases were decanted almost quantitatively and transferred to a second vial. Equal amounts of d_5-2AG (9.7 μL of a 100 pg/μL ethanolic solution) and d_4-AEA (9 μL of a 100 pg/μL ethanolic solution) were added to the extracts and the solvents were evaporated to dryness by a gentle nitrogen stream. Residues were then taken up with 30-μL aliquots of water–methanol (1:3, v/v) and 25-μL aliquots were injected into the UPLC–MS/MS system. Analyses were performed as described above and extraction yield was calculated by dividing the peak areas of d_8-2AG and d_8-AEA by the peak areas of d_5-2AG and d_4-AEA, respectively, and by multiplying with 100%. All extractions were performed in duplicate.

2.3.3. Studies on matrix-effect-causing phospholipids

Plasma phospholipids are well known to be one of the major contributors to matrix-effects in LC–MS/MS [17]. The above mentioned extraction procedures were applied to 250-μL aliquots of pooled unspiked human plasma. Aliquots (25-μL) of the final water–methanol (1:3, v/v) solutions were injected and analyzed by SRM according to the recommendations by Xia et al. [25] (see Table 3) in order to quantify the matrix-effect. The chromatograms from the SRM of LysoPC (16:0) and LysoPC (18:0), i.e., *m*/*z* 496.1 → *m*/*z* 184.1 and *m*/*z* 524.4 → *m*/*z* 184.1, provided the most intense peaks in all examined extracts. The summation of the peak areas of their five largest peaks were therefore chosen for quantitative comparison.

2.3.4. Studies on 2AG/1AG isomerisation

In experiments involving solvent evaporation of ethanolic 2AG and 1AG solutions we observed that 2AG was almost entirely isomerized to 1AG, whereas 1AG did not isomerize to 2AG. We therefore investigated whether 2AG isomerisation may depend upon the organic solvent used. Dilutions (100-μL aliquots) of 100 nM 2AG were freshly prepared in triplicate in different solvents and put on ice. The tested solvents were acetone, acetonitrile, chloroform, chloroform–methanol (2:1, v/v), ethanol, ethyl acetate, ethyl acetate–heptane (1:9, v/v), ethyl acetate–isohexane (1:1, v/v), isohexane, methanol, toluene, and water for comparison. Immediately thereafter, solvents were evaporated to complete dryness under a gentle nitrogen stream. Each sample vial was removed from the nitrogen evaporator as soon as no liquid was visible anymore. The evaporation process took 4 min for isohexane, 14 min for ethyl acetate and toluene, and almost 1 h for water. Residues were reconstituted in 100-μL aliquots of water–methanol (1:3, v/v) and analyzed immediately by UPLC–MS/MS as described above. As a

Table 3
SRM and corresponding phospholipid classes for quantifying matrix effects in LC–MS/MS after Xia et al. [24].

SRM	Phospholipid class short name	Phospholipid class long name
ESI−		
m/*z* 409.2 to *m*/*z* 153.1	LysoPA (C16:0)	Lysophosphatidic acid (C16:0)
m/*z* 511.3 to *m*/*z* 153.1	LysoPG (C18:0)	Lysophosphatidylglycerol (C18:0)
m/*z* 522.3 to *m*/*z* 153.1	LysoPS (C18:1)	Lysophosphatidylserine (C18:1)
m/*z* 834.4 to *m*/*z* 153.1	PS (C18:0/C22:6)	Phosphatidylserine (C18:0/C22:6)
m/*z* 861.6 to *m*/*z* 153.1	PI (C18:1/C18:1)	Phosphatidylinositol (C18:1/C18:1)
m/*z* 861.6 to *m*/*z* 241.2	PI (C18:1/C18:1)	Phosphatidylinositol (C18:1/C18:1)
ESI+		
m/*z* 454.3 to *m*/*z* 313.3	LysoPE (C16:0)	Lysophosphatidylethanolamine (C16:0)
m/*z* 496.1 to *m*/*z* 184.1	LysoPC (C16:0)	Lysophosphatidylcholin (C16:0)
m/*z* 524.3 to *m*/*z* 339.3	LysoPS (C18:0)	Lysophosphatidylserine (C18:0)
m/*z* 524.4 to *m*/*z* 184.1	LysoPC (C18:0)	Lysophosphatidylcholin (C18:0)
m/*z* 731.6 to *m*/*z* 184.1	SM (dC18:1/C18:0)	Sphingomyelin (dC18:1/C18:0)
m/*z* 758.5 to *m*/*z* 184.1	PC (C16:0/C18:2)	Phosphatidylcholine (C16:0/C18:2)
m/*z* 760.5 to *m*/*z* 184.1	PC (C16:0/C18:1)	Phosphatidylcholine (C16:0/C18:1)
m/*z* 772.6 to *m*/*z* 184.1	PL PC (C18/C18:1)	Plasmalogen phosphatidylcholin (C18:0/C18:1)

control, 100-μL aliquots of freshly prepared 100-nM 2AG solutions in water–methanol (1:3, v/v) were analyzed by UPLC–MS/MS without evaporation or dilution. The areas of the peaks of 2AG (retention time, 4.1 min) and 1AG (retention time, 4.4 min) were determined and used to calculate the peak area ratio as well as the sum of the peak areas of 2AG and 1AG.

2.3.5. Quantification of 2AG and AEA in human plasma

The following protocol was used for plasma samples from clinical studies and quality control (QC) samples on a routine basis. Blood from a forearm vein was drawn into a K^+-EDTA containing vial and immediately centrifuged (4655 × *g*, 4 °C, 10 min). Plasma aliquots (1 mL placed in a 2.5 mL plastic tube) were stored at −80 °C until analysis. After thawing on ice, each sample was spiked with an 18-μL aliquot of an ethanolic solution of the internal standards d_5-2AG (53.3 pg/μL) and d_4-AEA (50 pg/μL) to reach final concentrations of 2.5 nM each, and the plasma sample was incubated for 15 min on ice. Solvent extraction was performed by adding toluene (1 mL) to each sample and by shaking twice in a Precellys® 24 Dual Homogenisator at 5000 rpm for 20 s each with an interruption of 5 s. With these settings, no warming of the samples was observed. Phase separation was achieved by centrifugation (4655 × *g*, 4 °C, 5 min). The upper organic phase was transferred to a 1.5-mL glass vial and evaporated at room temperature (about 25 °C) to dryness under nitrogen. To the residue a 40-μL aliquot of water-methanol (1:3, v/v) was added and mixed by vortexing for 10 s. A 35-μL aliquot of the solution was then transferred to a second vial, from which a 25-μL aliquot was analyzed by UPLC–MS/MS as described above.

2.3.6. Method validation

Human pooled plasma from the local blood donor bank was used for method validation of d_0-2AG and d_0-AEA for added concentrations of 0 nM, 0.5 nM, 5 nM and 20 nM each on two consecutive days. All samples were spiked with the internal standards d_5-2AG (2.5 nM) and d_4-AEA (2.5 nM) and analyzed in triplicate. The methods accuracy (recovery, %), imprecision (RSD, %), lower limit of quantification (LLOQ) and relative lower limit of quantification (rLLOQ) were calculated as described [26].

The UPLC–MS/MS method was cross-validated for AEA in human plasma by means of a previously reported GC–MS/MS method for plasma AEA [15]. For this, 250 different plasma samples from healthy humans generated in a clinical study were analyzed by UPLC–MS/MS as described here and by GC–MS/MS which is also based on toluene solvent extraction of AEA from plasma [15]. It is noteworthy that the complete UPLC–MS/MS analyses took place about one year later than the GC–MS/MS analyses.

2.4. Quality control

During routine plasma sample measurements from several clinical studies, each batch of prepared samples was analyzed alongside four QC samples. One sample (QC1) was unspiked pooled plasma obtained from the blood bank. The other three QC plasma samples were spiked each with 0.5 nM (QC2), 1.0 nM (QC3) and 2.0 nM (QC4) of d_0-AEA and d_0-2AG. Sample preparation and UPLC–MS/MS analysis were carried out as described above.

2.5. Studies on the occurrence of 2AG and AEA in human urine

The applicability of the UPLC–MS/MS method originally developed for plasma 2AG and AEA was tested for human urine. Aliquots (5 mL) of urine samples from 19 healthy volunteers were extracted with toluene (5 mL) by means of a vortex mixer and further processed as described for plasma and analyzed by SRM. Urinary creatinine was determined by GC–MS as described [27]. 2AG and AEA concentrations in urine were divided by the creatinine concentration measured in the urine, and excretion rates were expressed as pmol 2AG or AEA per mmol creatinine.

2.6. Statistical analysis

If not otherwise specified, quantitative analyses were performed in triplicate. Values are presented as mean ± standard deviation. Linear regression was performed without weighting.

3. Results

3.1. LC–MS and LC–MS/MS analysis of 2AG, 1AG, and AEA

Baseline separation of 2AG and AEA was achieved on reverse phase material using various gradient elution forms with mobile phases A and B (data not shown). By contrast, separation of 2AG and 1AG was by far more challenging. Best separation was achieved using a very flat gradient from 75% methanol to 79% methanol over 4.5 min. A larger portion of water in the mobile phase resulted in peak broadening and loss of sensitivity, whereas a larger methanol portion disproved chromatography. In order to reach baseline separation of 2AG and 1AG, a 100-mm long column was required and used. To keep the total analysis time short, the column was thermostated at 60 °C. The final method provided symmetric peaks and retention times of 3.7 min for AEA, 4.1 min for 2AG, and 4.4 min for 1AG (Fig. 2). No differences in retention times were noted for all pairs of unlabeled and deuterium-labeled compounds.

The UPLC–MS ESI+ spectra of d_0-AEA, d_4-AEA, d_0-2AG and d_5-2AG contained both protonated molecules and those adducted with Na^+ and K^+ (Fig. 3). The most abundant ions for d_0-AEA and d_4-AEA were *m/z* 348.4 and *m/z* 352.4 ($[M+H]^+$), respectively. Less abundant ions (about 60–80% compared to the $[M+H]^+$ ions) were at *m/z* 370.4 (d_0-AEA) and *m/z* 374.4 (d_4-AEA) due to $[M+Na]^+$, and at *m/z* 386.4 (d_0-AEA) and *m/z* 390.4 (d_4-AEA) due to $[M+K]^+$. The mass spectra of d_0-2AG and d_5-2AG contained ions at *m/z* 379.4 and *m/z* 384.4 ($[M+H]^+$), *m/z* 401.4 and *m/z* 406.4 ($[M+Na]^+$), and *m/z* 417.4 and *m/z* 422.4 ($[M+K]^+$), respectively. The most abundant ions in

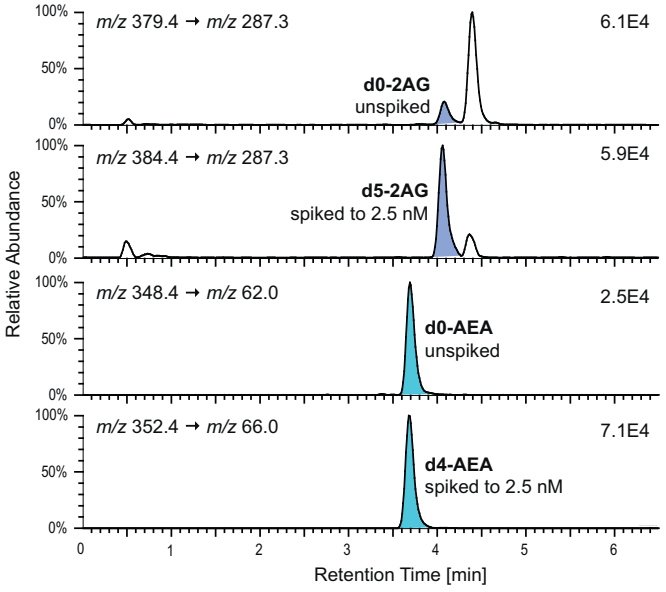

Fig. 2. Typical UPLC–MS/MS ESI+ chromatograms from a toluene-extracted plasma sample spiked with the internal standards d_5-2AG (2.5 nM) and d_4-AEA (2.5 nM). The non-filled peak eluting shortly behind d_0-2AG and d_5-2AG is due to the isomers d_0-1AG (endogenous) and d_5-1AG (internal standard).

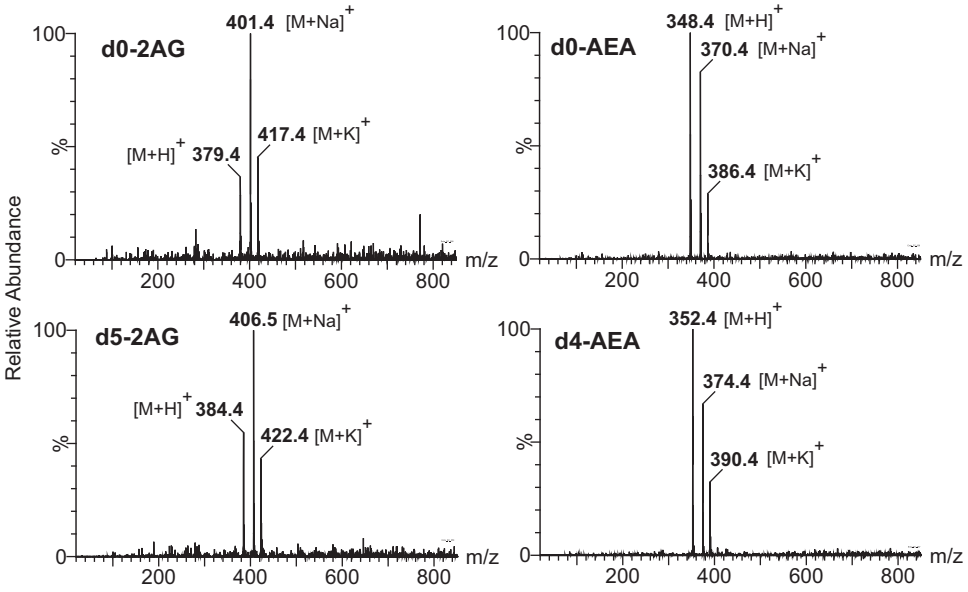

Fig. 3. UPLC–MS ESI+ spectra generated by injecting 5 ng of each d_0-2AG (upper left), d_0-AEA (upper right), d_5-2AG (lower left), and d_4-AEA (lower right). Full-scan data was recorded in the range from m/z 20 to m/z 850 with a scan rate of 0.5 scans/s.

the UPLC–MS ESI+ spectra of 2AG and 1AG were due to $[M+Na]^+$ followed by the $[M+H]^+$ and $[M+K]^+$ ions. Addition of ammonium acetate (2 mM) to the mobile phases shifted the pattern in favour of the $[M+H]^+$ ions. However, neither ammonium acetate nor ammonium formate, formic acid or acetic acid made the $[M+H]^+$ ions of 2AG to the most intense ions compared to $[M+Na]^+$ and $[M+K]^+$. Methanol and acetonitrile, tested as the organic modifier in the mobile phases, did not influence the pattern of $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$. We observed a consistent intensity pattern of these ions when 2-mM ammonium acetate-containing mobiles phases were prepared daily. Furthermore, we found that the ions $[M+Na]^+$ and $[M+K]^+$ of AEA and 2AG did not fragment in the collision-induced dissociation (CID) mode with argon as the collision gas over the entirely available range of collision energy (i.e., from 0 eV to 120 eV).

The UPLC–MS/MS ESI+ spectra obtained from CID of the $[M+H]^+$ ions of unlabeled and deuterium-labeled 2AG, 1AG and AEA were very similar to those previously reported by others [13,24]. Hence, the most intense product ions were m/z 62 from m/z 348.4 for d_0-AEA, m/z 66 from m/z 352.4 for d_4-AEA, m/z 287.3 from m/z 379.4 for d_0-2AG and m/z 287.3 from m/z 284.4 for d_5-2AG. These mass transitions were used for quantitative analysis of these substances in the SRM mode (Table 2).

3.2. Solvent extraction and matrix-effects

All organic solvents used for the solvent extraction of 2AG and AEA from human plasma provided similar extraction yields ranging between 70% and 80% for 2AG and 60% to 70% for AEA (Fig. 4).

UPLC–MS/MS analysis of the extracts obtained from solvent extraction with six different organic solvents or solvent mixture resulted in different chromatograms. Interestingly, SRM for the selected phospholipids did not reveal a single peak eluting with the retention time of 2AG or AEA (Figs. 5 and 6).

The UPLC–MS/MS chromatograms acquired in the ESI− mode, which is used to analyse PI, PS, LysoPS, LysoPG and LysoPA [25], only showed peaks when plasma was extracted by the “Folch” or the “Bligh & Dyer” procedures, ethyl acetate or ethyl acetate–heptane. The largest peaks were found from analysis of “Folch” extracts for LysoPA 16:0 (at 3.4 min); however the intensity of the signals was relatively low. Other phospholipids measured in the ESI− mode appeared as peaks at the same retention time but to a lesser extent. Injection of toluene and ethyl acetate–isohexane extracts resulted in chromatograms with no visible peaks from SRM of these six mass transitions (Fig. 6).

Chromatograms acquired in the ESI+ mode, which is used to analyse PL PC, PC, SM, LysoPC, LysoPS and LysoPE [25], showed

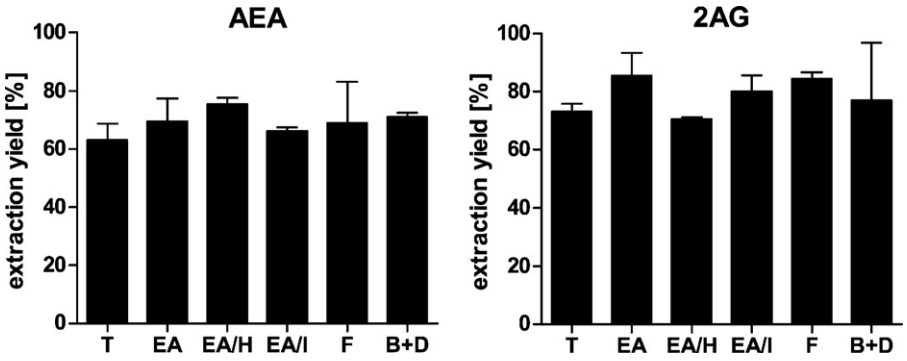

Fig. 4. Extraction yields acquired with different organic solvents used for solvent extraction, i.e., toluene (T), ethyl acetate–isohexane 1:1 (EA/I), ethyl acetate (EA), ethyl acetate–heptane 1:9 (EA/H), Bligh & Dyer (BD) and Folch (F). Error bars represent the range; all samples were prepared and analyzed in duplicate. UPLC–MSMS ESI+ was used.

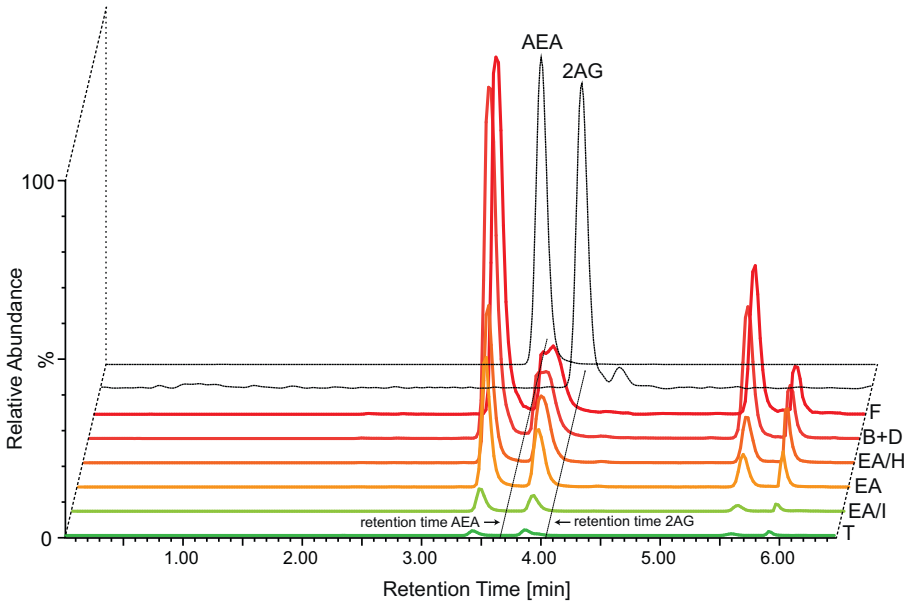

Fig. 5. Total ion current (TIC) chromatogram of the LysoPC (C18:0), i.e., m/z 524.4 → m/z 184.1, and the LysoPC (C16:0), i.e., m/z 496.1 → m/z 184.1, for all six examined solvent extracts, i.e., toluene (T), ethyl acetate-isohexane 1:1 (EA/I), ethyl acetate (EA), ethyl acetate–heptane 1:9 (EA/H), Bligh & Dyer (BD) and Folch (F). For comparison, the vertical scale is the same for each chromatogram, for easier viewing ability the chromatograms are set apart from each other by about 3% in vertical and 1% in horizontal direction. The black lines represent chromatograms acquired from injection of synthetic AEA and 2AG to show the retention time of the two peaks. *Note*: the vertical scale is not comparable to those of the TIC chromatograms.

distinct peaks at several retention times, often more than one peak per mass transition monitored. The most abundant signal was acquired from the LysoPC 16:0 with peaks at 3.4 min and 3.9 min and peak areas as large as 14×10^6 (arbitrary units). Other large peaks were found in the LysoPC 18:0 chromatograms at retention times of 4.0, 4.6 and 5.9 min with peak areas up to the order of 5×10^6 (arbitrary units). The sum of the five peak areas for each of the six extracts is shown in Fig. 7 for the purpose of comparison. The "Folch" and the "Bligh & Dyer" extracts obviously contain the largest amounts of matrix-effect-causing phospholipids. On the other hand, ethyl acetate–heptane or ethyl acetate extracts contain moderate amounts of phospholipids, i.e., 43% and 25%, respectively, with regard to the "Folch" extracts. The lowest amount of phospholipids extracted from plasma was found in toluene extracts (2%) followed by ethyl acetate–isohexane extracts (5%).

3.3. Isomerisation of 2AG to 1AG

UPLC–MS/MS analysis of the control sample, i.e., the 2AG solutions that had not been evaporated to dryness prior to reconstitution in water–methanol (1:3, v/v), resulted in a 2AG/1AG peak area ratio of 14:1, indicating that the sample contained about 93% 2AG and 7% 1AG. This value is very close to the value of 10% 1AG declared by the manufacturer for the 2AG solution. Interestingly, the same result was obtained using toluene as the solvent for 2AG/1AG, indicating that no additional isomerisation of 2AG to 1AG took place during toluene evaporation.

For all other tested solvents, the mean 2AG/1AG ratio was smaller than 14:1. In particular, the 2AG/1AG ratio measured was 12.3:1 for chloroform, 10.8:1 for isohexane, 9.8:1 for ethyl acetate, 9.5:1 for ethyl acetate–heptane (1:9, v/v), 8.1:1 for water, 7.5:1 for ethyl acetate–isohexane (1:1, v/v), 6.9:1 for acetonitrile, 4.4:1 for chloroform–methanol (2:1, v/v), 4.1:1 for acetone, 2.6:1 for methanol, and only 0.07:1 for ethanol (see Fig. 8A and C for selected chromatograms). The value obtained for the ethanolic solution of 2AG is remarkably low, indicating that 2AG was converted almost quantitatively to 1AG within only 5 min, i.e., the time needed for ethanol evaporation.

The sum of the 2AG and 1AG peak areas in the individual samples was compared with the sum of the 2AG and 1AG peak areas measured in the control sample (Fig. 8B). The lowest values were obtained for water (35%) and methanol (72%), indicating that in these solvents 2AG and 1AG underwent degradation to a considerable degree. For the other solvents, the total peak area ranged between 130% and 150% of the control. In case of ethanol, isomerisation of 2AG to 1AG seems to have been taken place without appreciable loss of 2AG or 1AG due to degradation processes. Interestingly, toluene not only prevented 2AG isomerisation to 1AG, but it also avoided degradation of 2AG and 1AG (Fig. 8B).

3.4. Standardisation of internal standards

Linear regression analysis between the measured peak area ratio of deuterium-labeled to unlabeled standard (y) versus the amount of unlabeled standard injected (x, in pg) resulted in straight lines with regression equations: $y_1 = 1.86 + 0.46x_1$ ($r^2 = 0.999$) for d_5-2AG, and $y_2 = -22.4 + 1.31x_2$ ($r^2 = 0.998$) for d_4-AEA. Because the slope values deviated from the theoretical value of 1.00, the declared concentrations of d_5-2AG (53 pg/μL) and d_4-AEA (50 pg/μL) were corrected by dividing these concentrations by the respective slope value. Discrepancies between declared and measured concentrations of commercially available stable-isotope labeled substances are common and should be tested prior to use in quantitative analysis [28].

3.5. Analytical performance and method validation

Calculation of measured d_0-AEA concentrations [d_0-AEA] was performed by multiplying the concentration of the internal standard [d_4-AEA] by the measured peak area (PA) ratio PA(d_0-AEA)/PA(d_4-AEA) as described by Formula (F1):

$$[d_0\text{-AEA}] = [d_4\text{-AEA}] \times \frac{\text{PA}(d_0\text{-AEA})}{\text{PA}(d_4\text{-AEA})} \quad \text{(F1)}$$

Because the extent of isomerisation of 2AG to 1AG may be different for endogenous and/or added unlabeled 2AG and in the internal

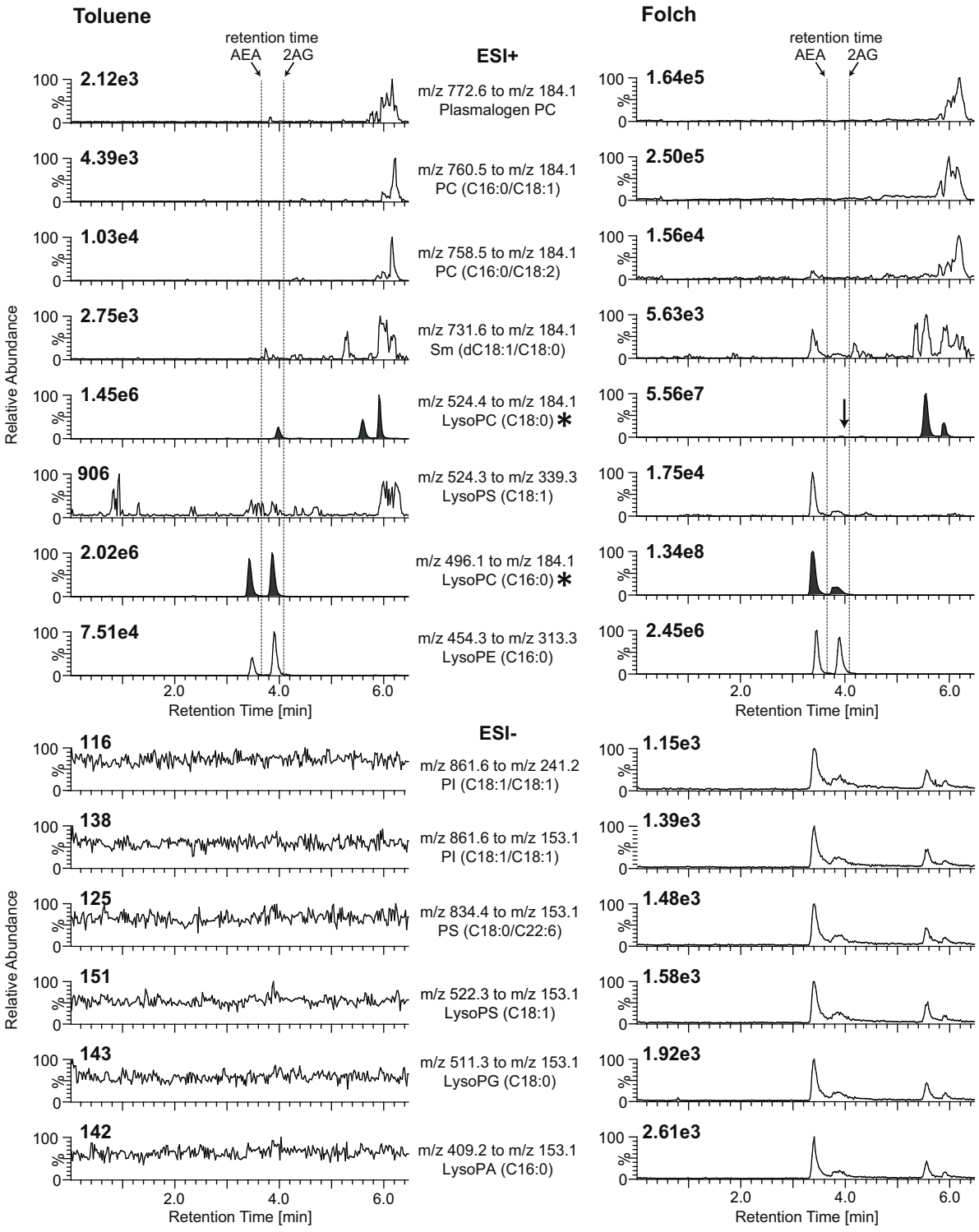

Fig. 6. Chromatograms of all recorded SRM for quantification of the matrix-effect-causing phospholipids, exemplarily from a toluene extract (left panel) and a Folch extract (right panel). The SRM indicated with an asterisk displayed the most abundant peaks in all extracts so that the filled peaks were chosen for integration and quantitative comparison. The arrow on the right panel indicates the retention time of the third peak in the chromatogram, which is too small to be visible compared to the two peaks that elute later. The bold numbers next to the vertical scale represent the respective SRM signal level of 100%, therefore allowing for comparison of the signals intensity for each chromatogram.

standard d_5-2AG, we used the following Formula (F2) for calculation of d_0-2AG concentrations:

$$[d_0\text{-2AG}] = [d_5\text{-2AG}] \times \left(\frac{(\text{PA}(d_0\text{-2AG})}{\text{PA}(d_5\text{-2AG}) + \text{PA}(d_5\text{-1AG})} \right) \quad \text{(F2)}$$

Formulas (F1) and (F2) were used to calculate [d_0-AEA] and [d_0-2AG] in the validation experiments. Table 4 summarizes the measured concentrations as well as the recovery and imprecision values from these experiments. 2AG and AEA were measured with an accuracy (recovery) of the order 100 ± 20% and an imprecision (RSD) below 20%, fulfilling generally acceptable ranges. Linear

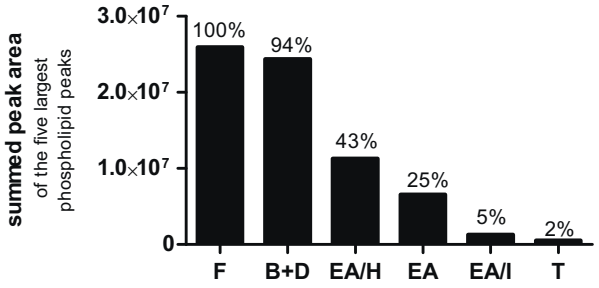

Fig. 7. Comparison of the peak areas summation of the five largest phospholipid peaks (shown in Fig. 6 as filled peaks) as representatives for the overall matrix-effect. The peaks used evolve from the chromatograms derived from LysoPC (C18:0), i.e., m/z 524.4 → m/z 184.1, and LysoPC (C16:0), i.e., m/z 496.1 → m/z 184.1.

regression analysis between measured d_0-AEA (y_1) or d_0-2AG (y_2) concentration and added d_0-AEA (x_1) or d_0-2AG (x_2) concentration resulted in the regression equations $y_1 = 1.11 + 1.01x_1$ ($r^2 = 0.997$) and $y_2 = 2.14 + 0.92x_1$ ($r^2 = 0.98$). These data indicate high linearity, mean recovery rates (see slope value) of 101% for d_0-AEA and 92% for d_0-2AG, and basal concentrations (see y-axis intercept value) of 1.11 nM for d_0-AEA and 2.14 nM for d_0-2AG in the pooled plasma used for method validation. The LOQ value of the method was each 0.5 nM for AEA and 2AG in human plasma. The rLLOQ is calculated by dividing the LOQ value by the basal concentration of the analyte in the matrix [26]. The rLLOQ value of the method was calculated as 45% for AEA and 23% for 2AG. Typical UPLC–MS/MS ESI+ chromatograms from quantitative analyses of 2AG and AEA in human plasma are shown in Fig. 2.

Table 4
Precision and accuracy (recovery, %) of the LC–MS/MS method for 2AG and AEA in human plasma.[a]

Added (nM)	Measured (nM) (mean ± SD)	Precision (RSD, %)	Recovery (%)
2AG			
0.0	2.04 ± 0.08	4.03	n.a.
0.5	2.54 ± 0.38	14.8	99.3
5.0	6.92 ± 0.63	9.16	97.5
20.0	20.47 ± 1.46	7.15	92.1
AEA			
0.0	1.05 ± 0.07	6.98	n.a.
0.5	1.57 ± 0.06	3.54	104.1
5.0	6.28 ± 0.20	3.10	104.6
20.0	21.21 ± 0.81	3.80	100.8

[a] Samples were prepared in triplicate on two consecutive days. Data were calculated from all six analyzed samples for each concentration. n.a. not applicable.

The LOD for AEA was achieved from injection of 20 μL of a solution containing 0.0128 pg/μL d_0-AEA and was determined as 0.256 pg d_0-AEA. The LOD for d_0-2AG was achieved from injection of 20 μL of a solution containing 0.32 pg/μL d_0-2AG and was determined as 6.4 pg d_0-2AG.

The present UPLC–MS/MS method was cross-validated by a previously described GC–MS/MS method for plasma AEA which uses the same toluene solvent extraction [14]. Linear regression analysis between UPLC–MS/MS (y) and GC–MS/MS (x) revealed a close correlation ($r^2 = 0.821$) with the regression equation being $y = 0.01 + 0.85x$. Differences between the methods may be in part

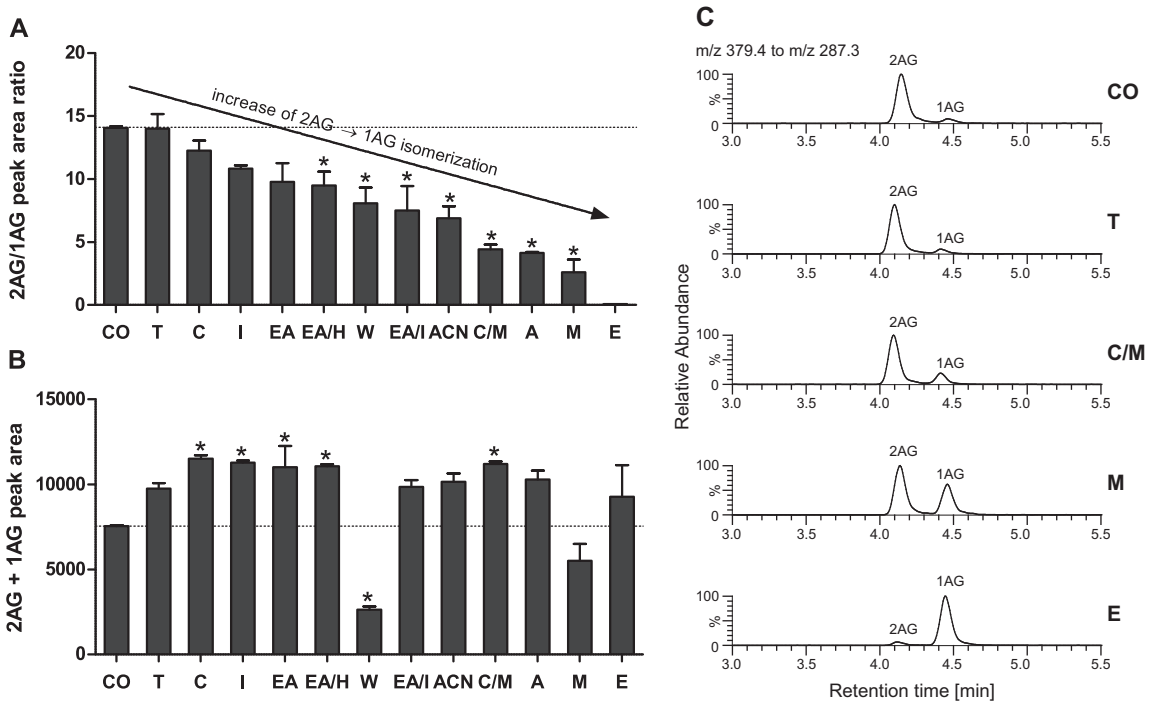

Fig. 8. (A) Comparison of acquired 2AG/1AG peak area ratios after evaporation of 100-μL aliquots of a 100-nM 2AG solution in different organic solvents (Control (CO), toluene (T), chloroform (C), isohexane (I), ethyl acetate (EA), ethyl acetate–heptane 1:9 (EA/H), water (W), ethyl acaetate–isohexane 1:1 (EA/I), acetonitrile (ACN), chloroform–methanol 2:1 (C/M), acetone (A), methanol (M), ethanol (E)), resuspension in 100-μL aliquots of water–methanol (1:3, v/v) and injection into the UPLC–MS/MS system. Control samples consisted of 100 μL of a 100 nM 2AG solution in water–methanol (1:3, v/v). (B) Summed areas of the 2AG and 1AG peaks for the corresponding extract shown above for examining putative degradation processes. All samples were prepared in triplicate, error bars represent the standard deviations, and asterisks indicate a statistically significant difference compared to the control samples according to a one-way ANOVA analysis with 95% confidence intervals (A and B). (C) Selected sample chromatograms for 2AG and 1AG acquired after evaporation of 100-μL aliquots of a 100-nM 2AG solution in different organic solvents (toluene (T), chloroform–methanol 2:1 (C/M), methanol (M) and ethanol (E)), reconstitution in 100-μL aliquots of water–methanol (1:3, v/v) and injection into the UPLC–MS/MS system. The control sample chromatogram (C) was obtained from analysis of a 100-μL aliquot of a 100-nM 2AG solution in water-methanol (1:3, v/v). All analyses were performed in the positive ESI mode.

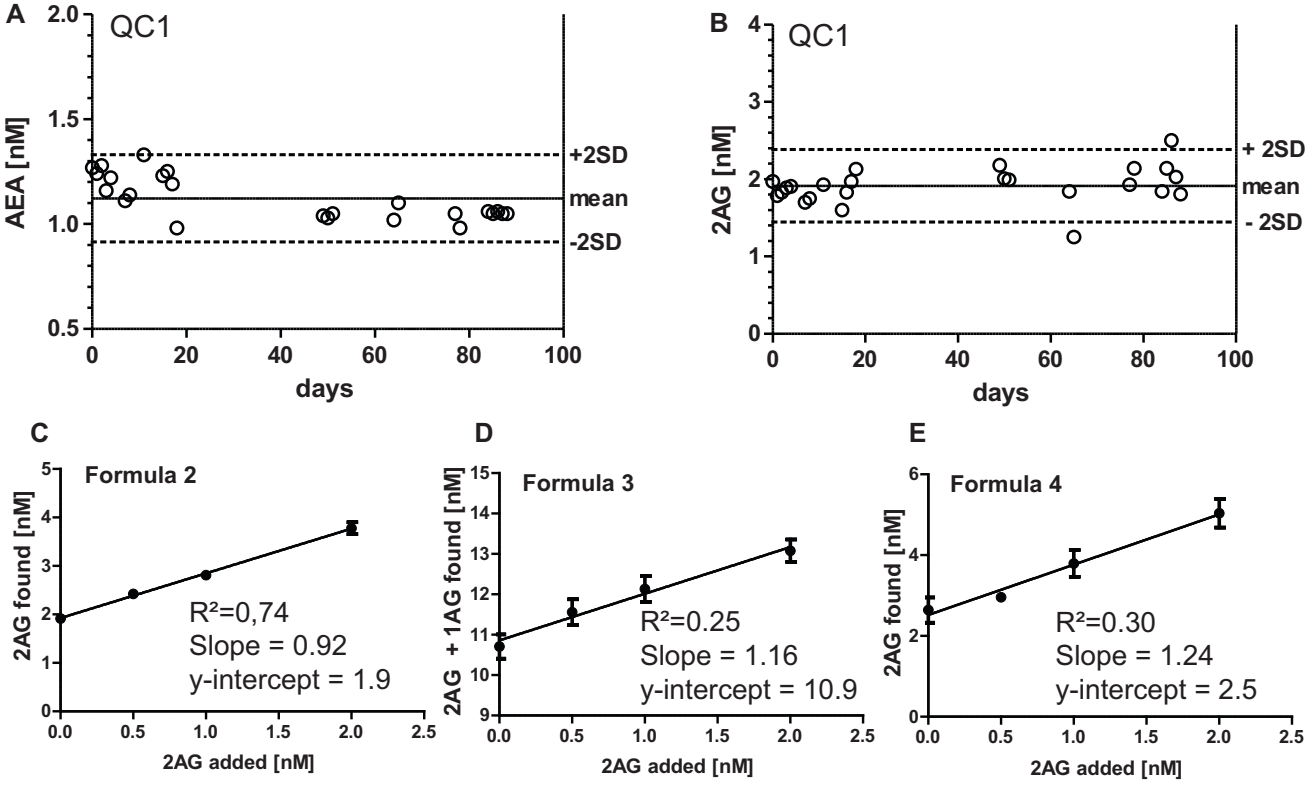

Fig. 9. QC charts from routine analysis of AEA (A) and 2AG (B) in human plasma by UPLC–MS/MS ESI+. The figures show 24 samples each, consisting of unspiked pooled plasma, that have been prepared during routine measurements as described in Section 2.6. Additionally, QC samples spiked to 0.5 nM, 1.0 nM and 2.0 nM of 2AG were prepared and are shown as calibration curves of 2AG concentrations by using different calculation modes. See Sections 3.5 and 3.6 for details on the formulas used. The figures show linear regression analysis with error bars representing the standard error (C–E).

due to the fact that UPLC–MS/MS analyses took place about one year later than the GC–MS/MS analyses.

3.6. Quality control

In total, 96 QC plasma samples were analyzed simultaneously for 2AG and AEA along with plasma samples from different clinical studies within a period of 100 days after sample preparation by a single laboratory technician. Fig. 9 shows the QC charts for 24 unspiked plasma samples for 2AG and AEA. For 2AG, 2 out of 24 concentrations were outside of the $\pm 2 \times$ SD regime. The mean 2AG concentration was 1.9 nM (RSD, 12.2%) (Fig. 9B). For AEA, all concentrations were within the $\pm 2 \times$ SD regime, the mean AEA concentration was 1.2 nM (RSD, 9.3%) (Fig. 9A). These data indicate remarkable stability of 2AG and AEA and satisfactory method precision for at least 100 days when plasma samples are stored frozen at −70 °C. The AEA stability from UPLC–MS/MS analyses confirms the AEA stability in human plasma previously observed by GC–MS/MS [15].

3.7. Quantification modes for 2AG

By using the data of all 24 QC samples we tested the validity of Formula (F2) and in addition the validity of Formula (F3) (see below) which is commonly used for calculation of 2AG concentrations from LC–MS/MS analyses [12,13,28]. Fig. 9C shows a reasonable linearity ($r^2 = 0.74$) and y-axis intercept and slope values of 1.9 nM and 0.92, respectively, when Formula (F2) is used. By contrast, Formula (F3) yielded analytically unacceptable linearity ($r^2 = 0.25$), and a much higher y-axis intercept of 10.9 nM (Fig. 9D). Finally, we tested whether non-consideration of the isomerisation of the internal standard d_5-2AG may yield reliable 2AG concentrations in plasma samples (Formula (F4)). As shown in Fig. 9E, this mode leads to weak linearity ($r^2 = 0.30$), but reasonable values for slope (1.24) and y-axis intercept (2.5 nM). In conclusion, this comparison demonstrates that most valid 2AG concentrations in human plasma are obtained by using Formula (F2).

$$[d_0\text{-2AG}] = [d_5\text{-2AG}] \times \left(\frac{\text{PA}(d_0\text{-2AG}) + \text{PA}(d_0\text{-1AG})}{\text{PA}(d_5\text{-2AG}) + \text{PA}(d_5\text{-1AG})} \right) \quad \text{(F3)}$$

$$[d_0\text{-2AG}] = [d_5\text{-2AG}] \times \left(\frac{\text{PA}(d_0\text{-2AG})}{\text{PA}(d_5\text{-2AG})} \right) \quad \text{(F4)}$$

3.8. Occurrence of 2AG and AEA in human urine

AEA was not detectable in any urine sample of the 19 healthy subjects. 2AG was detected in 3 out of the 19 urine samples at concentrations of 160, 180 and 212 pM, corresponding to 12.3, 14.5 and 9.9 pmol/mmol creatinine. A representative UPLC–MS/MS chromatogram for urinary 2AG by the present method is shown in Fig. 10.

4. Discussion

2AG and 1AG are glycerol esters of arachidonic acid, whereas AEA is the ethanol amide of arachidonic acid (Fig. 1). 2AG, 1AG and AEA are electrically uncharged within a wide pH range. Previously, we have utilized this particular physicochemical property and used a single solvent extraction with toluene for the specific extraction of AEA from human plasma without pH correction and quantification by GC–MS/MS [15]. Others [24] have integrated solvent extraction with toluene of AEA from various matrices in a LC–MS/MS method that involved additional purification steps including thin-layer chromatography. However, this group was

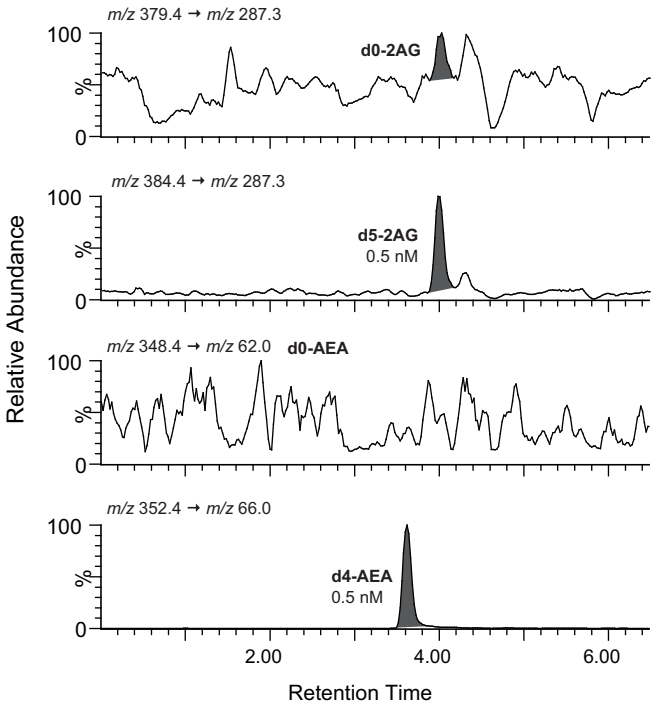

Fig. 10. UPLC–MS/MS chromatograms from the analysis of 2AG and AEA in a human urine sample. 2AG was detected in this urine sample at a concentration of 212 pM or 9.9 pmol/mmol creatinine.

unable to detect any AEA in human serum and plasma [24]. In toluene plasma extracts we also found 2AG and 1AG by GC–MS/MS, but reliable quantification of plasma 2AG and 1AG with this method was not possible because of the instability of the various derivatives of the glycerol moieties of 2AG and 1AG (unpublished observations). Given the promising results obtained from the use of toluene for 2AG, 1AG and AEA extraction, we were interested in testing the utility of the toluene extraction in the quantitative determination of 2AG and AEA in human plasma by the LC–MS/MS technology, currently the most widely applied methodology in the area of EC research [23,30]. Measuring these EC by LC–MS/MS is mostly combined with classical extraction methods including the "Folch" and the "Bligh & Dyer" methods until today [4,12,14,31]. Because 2AG quickly isomerizes to the biologically inactive 1AG, we were also interested in defining analytical conditions that avoid or minimize the extent of isomerisation. For this purpose we used a stable-isotope dilution UPLC–MS/MS method and solvent extraction with different solvents. We developed approaches that minimize matrix-effects caused by phospholipids, avoid isomerisation of 2AG to 1AG and enhance stabilization of 2AG. Our results suggest that all these achievements are largely due to toluene usage for solvent extraction. Other traditional extraction methods seem to be less useful in the LC–MS/MS analysis of 2AG, most likely because they allow abundant extraction of plasma phospholipids contributing to matrix-effects. Presumably, toluene extraction would also be useful for LC–MS/MS and GC–MS/MS analysis of other neutral lipids.

We noticed differences between AEA and 2AG in their LC–MS/MS properties in addition to their chromatography. On a molar basis, AEA provided approximately 3-fold larger peak areas than 2AG. Most likely, these differences originate from the more abundant protonation of the AEA amide group, whereas 2AG and 1AG form rather Na^+ and K^+ adducts (Fig. 3). This property is important because Na^+ and K^+ adducts of EA and 2AG are highly resistant against CID and cannot be used for quantification of AEA and 2AG by LC–MS/MS. For maximum sensitivity Na^+ and K^+ adduct formation needs to be kept to a minimum. This goal can be achieved by adding 2 mM ammonium acetate to the mobile phases and by avoiding Na^+ and K^+ contamination of all parts of the LC–MS/MS system. We realized this in our study by preparing mobile phases daily using freshly prepared distilled water in a regularly maintained Millipore system and by storing the mobile phases in low-volume glass flasks (<500 mL). Another difference between AEA and 2AG that may also contribute to smaller 2AG peak areas is the apparently lower stability of 2AG and its remarkable isomerisation to 1AG which elutes behind 2AG. Our results suggest that toluene extraction of AEA and 2AG from 1-mL plasma aliquots allows for simultaneous quantification of AEA, 2AG, and 1AG with analytically acceptable accuracy and precision (Table 4).

Most likely for traditional reasons [21,22], first published methods for AEA and 2AG involved the use of mixtures of chloroform and methanol, i.e., the so called "Folch" or "Bligh & Dyer" methods [32–34]. Alternative extraction methods include solvent extraction with toluene [15,24], ethyl acetate or ethyl acetate–heptane [6,13], solid-phase extraction (SPE) [7], or plasma protein precipitation with acetone [10]. Since 2AG and AEA are endogenous substances, and 2AG- and AEA-free plasma is not available, we applied the method proposed by Xia et al. [25] for quantifying matrix-effects caused by phospholipids. We evaluated different solvent extraction methods and excluded SPE and plasma protein precipitation procedures because they are known to generally cause matrix-effects due to co-extraction of phospholipids [18]. Expectedly, all examined solvents, including those in the "Folch" and "Bligh & Dyer" approaches and toluene, provided similar extraction yields for AEA (60–70%) and 2AG (70–80%). However, compared to solvent extraction with toluene, traditionally applied methods have several disadvantages: (1) the "Folch" and "Bligh & Dyer" methods are practically less feasible; (2) they require much larger organic solvent volumes, thus making them more expensive, environmentally harmful and time consuming; (3) more importantly, the "Folch" and "Bligh & Dyer" methods are associated with a 50-fold higher content of matrix-effects-causing phospholipids compared to toluene (Figs. 5–7); and finally (4) the "Folch" and "Bligh & Dyer" methods favour isomerisation of 2AG to 1AG. Taken together, solvent extraction of AEA, 2AG and 1AG from plasma with toluene seems to be the most reliable and simplest approach in their analysis by LC–MS/MS or GC–MS/MS.

An important issue is the isomerisation of 2AG to 1AG. It seems to take place spontaneously, with basic pH, elevated temperatures and presence of albumin being among the experimental parameters facilitating isomerisation [3]. Acidic conditions during protein precipitation with acetonitrile have been reported to attenuate 2AG/1AG isomerisation [8]. Moreover, isomerisation was found to be less abundant when acetonitrile [11], acetone or diethyl ether [35] is used instead of chloroform or methanol during sample preparation. Our study suggests that the evaporation process of 2AG-containing solutions in organic solvents plays a crucial role in the isomerisation process. Evaporation of ethanolic solutions of 2AG resulted in almost complete conversion of 2AG to 1AG, whereas evaporation of 2AG-containing toluene solutions yielded minimal 2AG to 1AG isomerisation (Fig. 8A). It seems that evaporation of 2AG solutions in polar and protic solvents is associated with a higher degree of isomerisation than non-polar and aprotic solvents such as toluene or isohexane. Remarkably, total AG content, i.e., the sum of 2AG and 1AG, is not decreased by the evaporation process of organic solvents (Fig. 8B). By contrast, evaporation of aqueous 2AG solutions resulted in loss of 2AG and 1AG, probably due to hydrolysis of these compounds to arachidonic acid and glycerol. Yet, the mechanisms of 2AG/1AG isomerisation and hydrolysis are still incompletely understood and demand further investigation.

To our surprise, we observed that UPLC–MS/MS analysis of the reconstituted extracts from most solvents resulted reproducibly in

values (peak areas) above those of the control samples. An explanation for this finding could be an ion-enhancement effect of the particular organic solvents. Whether this kind of ion-enhancement is a general phenomenon in LC–MS/MS, demands further examination. The requirement that the sample used as control in those investigations had not to be evaporated, may be an alternative explanation for the higher peak areas observed.

5. Conclusion

Toluene offers several analytically relevant advantages over traditionally applied organic solvents for AEA and 2AG extraction from human plasma and for quantitative LC–MS/MS analysis in the ESI+ mode: minimal matrix-effects because of a higher selectivity, minimal 2AG to 1AG isomerisation, and maximum 2AG stability, paired with a comparable extraction yield. Based on these findings, the usage of toluene instead of chloroform–methanol mixtures for solvent extraction of AEA and 2AG from plasma, urine and other relevant biological matrices is recommend. Combination of stable-isotope dilution with AEA and 2AG solvent extraction from plasma (1 mL) with toluene (1 mL) and UPLC–MS/MS ESI+ analysis allows for accurate, precise and fast quantification of AEA and 2AG in clinical settings. The extent of isomerisation of the deuterium-labeled 2AG needs to be considered in correctly calculating plasma concentrations of endogenous 2AG. 1AG in human plasma may exclusively originate from 2AG by isomerisation which may start with or even prior to blood sampling. Yet, the physiological and possibly pathological implications of 2AG and 1AG as well as their molar ratio found in different biological matrices remain to be investigated in the future. AEA seems not to occur in urine of healthy humans. 2AG measurement in plasma is superior to urine.

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