Analysis of ECs and related compounds in plasma: artifactual isomerization and ex vivo enzymatic generation of 2-MGs

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Abstract The analysis of peripheral endocannabinoids (ECs) is a good biomarker of the EC system. Their concentrations, from clinical studies, strongly depend on sample collection and time processing conditions taking place in clinical and laboratory settings. The analysis of 2-monoacylglycerols (MGs) (i.e., 2-arachidonoylglycerol or 2-oleoylglycerol) is a particularly challenging issue because of their ex vivo formation and chemical isomerization that occur after blood sample collection. We provide evidence that their ex vivo formation can be minimized by adding Orlistat, an enzymatic lipase inhibitor, to plasma. Taking into consideration the low cost of Orlistat, we recommend its addition to plasma collecting tubes while maintaining sample cold chain until storage. We have validated a method for the determination of the EC profile of a range of MGs and N-acyl ethanolamides in plasma that preserves the original isomer ratio of MGs. Nevertheless, the chemical isomerization of 2-MGs can only be avoided by an immediate processing and analysis of samples due to their instability during conservation. We believe that this new methodology can aid in the harmonization of the measurement of ECs and related compounds in clinical samples.—Pastor, A., M. Farré, M. Fitó, F. Fernandez-Aranda, and R. de la Torre. Analysis of ECs and related compounds in plasma: artificial isomerization and ex vivo enzymatic generation of 2-MGs. J. Lipid Res. 2014. 55: 966–977.

Supplementary key words 2-arachidonoylglycerol • 2-oleoylglycerol • validation • Orlistat • human • endocannabinoids • 2-monoacylglycerol

The neuromodulatory activities of the endocannabinoid (EC) system are involved in many human physiological and pathological functions (1–5). It comprises: i) two G-protein-coupled receptors, known as cannabinoid (CB)1 and CB2; ii) endogenous ligands for these two receptors, known as ECs, N-arachidonoyl ethanolamine [AEA (anandamide)] and 2-arachidonoylglycerol (AG) being the most studied; and iii) proteins that regulate EC tissue concentration (anabolic and catabolic enzymes), cellular distribution (EC-binding proteins and transporters), and CB receptor activity (CB receptor-interacting proteins) (1).

In addition to AEA and 2-AG, there are a number of structurally related compounds, also known as EC-related compounds (ERCs), derived from less unsaturated fatty acids: N-acyl ethanolamides (NAEs) such as N-linoleoyl ethanolamide (LEA), N-oleoyl ethanolamide (OEA), N-palmitoyl ethanolamide (PEA), N-palmitoleoyl ethanolamide, and N-stearoyl ethanolamide [STE, ethanolamide; QC, quality control; QC-H, quality control-high; QC-L, quality control-low; QC-M, quality control-mid; RF, response factor; SA, surrogate analyte; SEA, N-ethanolaminoethylglycerol; TBME, tert-butyl-methyl-ether.

Abbreviations: AEA, N-arachidonoyl ethanolamide; AG, arachidonylglycerol; Am. Ac., ammonium acetate; CB, cannabinoid; CV, coefficient of variation; DAG, diacylglycerol; DAGL, n-1-diacylglycerol lipase; DAEA, N-docosatetraenoyl ethanolamide; DGLEA, N-dihomo- ylinolenoyl ethanolamide; DHEA, N-docosahexaenoyl ethanolamide; EC, endocannabinoid; EPEA, N-eicosapentaenoyl ethanolamide; ERC, endocannabinoid-related compound; ISTD, internal standard; LDA, N-linoleoyl ethanolamide; α-LEA, N-linoleoyl ethanolamide; LG, linoleoylglycerol; LLOQ, lower limit of quantification; LOD, limit of detection; MG, monoacylglycerol; MRM, multiple reaction monitoring; NAE, N-acyl ethanolamide; OA, N-oleoyl ethanolamide; OG, oleoylglycerol; PEA, N-palmitoyl ethanolamide; POEA, N-palmitoleoyl ethanolamide; QC, quality control; QC-H, quality control-high; QC-L, quality control-low; QC-M, quality control-mid; RF, response factor; SA, surrogate analyte; SEA, N-ethanolaminoethylglycerol; TBME, tert-butyl-methyl-ether.

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2 www.jlr.org
3 The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of one table.
(POEA), and N-stearoyl ethanolamide (SEA), among others. Analogously, for the 2-monoacylglycerol (MG) series, there are also homologs such as 2-oleoylglycerol (OG) and 2-linoleoylglycerol (LG). Most of these molecules do not directly bind to CB receptors but may enhance/modify the actions of ECs (entourage effect) or display biological activities related to their interactions with other receptors such as GPR119 (OEA, POEA, LEA, 2-OG) (7, 8), transient receptor potential vanilloid type 1 (TRPV1) (OEA), GPR55 (PEA), or PPAR-ε (OEA) (9). Finally, two putative ECs, N-eicosapentaenoyl ethanolamide (EPEA) and N-docosahexaenoyl ethanolamide (DHEA) and N-docosahexaenoyl ethanolamide (DHEA), derived from the n-3 polyunsaturated fatty acids, are able to bind with low affinity to the CB1 and CB2 receptors and may have biological significance in the brain (10, 11).

A number of publications have been devoted to the analysis of ECs in plasma, and an effort has recently been made to establish reference intervals for five ECs in human plasma (12). As the chemistry and biology of ECs are better understood, so is the need to improve measurements in human plasma through greater control of factors that introduce variability. At present, such factors still limit the interchangeability of EC plasma concentrations from clinical studies. It has already been established that the chemical properties of ECs, with respect to their stability during analytical procedures (extraction solvents, pH conditions, and evaporation of organic solvents) and their absorption by glassware and plastic materials, are very relevant factors to take into consideration. A comprehensive review has provided a full discussion on these issues (13) and several analytical methods have already taken these factors into account (12, 14). Discrepancies among laboratories probably originate from preanalytical sample procurement protocols and compound-specific factors.

Concerning biological matrices, ECs are unstable in those where enzymes are involved in their synthesis and clearance: the fatty acid amide hydrolase hydrolyzes AEA to arachidonic acid and ethanolamine, and the MG lipase is responsible for the hydrolysis of 2-AG to arachidonic acid and glycero. Nevertheless, accumulated experience suggests that the incorporation of unspecific enzyme inhibitors of amidases, esterases, and proteases, such as PMSF, to sample collection tubes is not justified. However, blood-containing tubes not centrifuged immediately in cold conditions after withdrawal may cause artifactual exaggerated NAE concentrations due to ex vivo release of from erythrocytes or leukocytes (12). Additionally, it has been reported that ex vivo synthesis of 2-AG for plasma preserved at room temperature and abundant 2-AG/1-AG isomerization is due to sample analysis conditions (12, 14). The two main ECs, AEA and 2-AG, are produced from different biosynthetic pathways. AEA is generated from N-arachidonoyl phosphatidylethanolamines by several possible biosynthetic routes with multiple enzymes implicated: the N-acyl phosphatidylethanolamine-specific phospholipase D, the α,β-hydrolyase-4 (ABHD4), the glycerophosphodiesterase-1 (GDE1), a soluble phospholipase A2, an unidentified phospholipase C, and phosphatases (15). In contrast, the biosynthetic precursors for 2-AG, the sn-1-acyl-2-AGs, are mostly produced by phospholipase Cβ acting on membrane phosphatidylinositol, and then being converted to 2-AG by the action of either of two isoforms of the same enzyme, the sn-1-diacylglycerol lipases α and β (DAGLα and DAGLβ) (15, 16).

In clinical studies, the determination of ECs and ERCs is limited by methodological issues which particularly concern 2-MGs. Both their chemical isomerization and ex vivo generation are major issues that limit their inclusion as disease/physiological biomarkers. The aim of the present work is to improve current available methodological approaches for a better understanding of the biological significance of ECs.

MATERIALS AND METHODS

Chemicals and laboratory material

Ammonium acetate (Am. Ac.), acetic acid, tert-butyl-methyl-ether (TBME), acetonitrile, and formic acid were from Merck (Darmstadt, Germany). 1-AG, 1-AG-d5, 2-AG, 2-AG-d5, 2-AG-d8, 2-LG, 1-LG, AEA, AEA-d4, AEA-d8, N-docosatetraenoyl ethanolamide (DEA), N-dihomo-γ-linolenoyl ethanolamide (DGLEA), DHEA, DHEA-d4, LEA, LEA-d4, PEA, PEA-d4, POEA, POEA-d4, OEA, OEA-d4, and SEA were from Cayman Chemical (Ann Arbor, MI). 1-OG and 2-OG were from Sigma-Aldrich (St. Louis, MO). 1-OG-d5 and 2-OG-d5 were from Toronto Research Chemicals (North York, ON, Canada). FIPI hydrochloride (CAS 990955-18-2), D609 (CAS 83373-60-8), edelfosine (CAS 77286-66-9), and GSK 264220A (CAS 685506-42-7) were from Tocris Bioscience (Bristol, UK). Orlistat (tetrahydrolipstatin) was from Cayman Chemical. KT172, KT109, and RHC 80267 (CAS 83654-05-1) were from Sigma-Aldrich. KIMAX 16 × 125 mm screw cap glass borosilicate tubes were from Kimble Chase (Mexico). Nunc 1.8 ml cryotube vials were from Thermo Fisher Scientific (Roskilde, Denmark). Ultrapure deionized water was produced by a Milli-Q Advantage A10 system from Millipore (Madrid, Spain).

Standard solutions

The purity of the NAE standards AEA, DEA, DGLEA, DHEA, DHEA, LEA, PEA, PEA, POEA, POEA, OEA, and SEA was >98% as provided by the manufacturer. Purity of the MG standards was >95% for 2-AG, 1-AG, and 2-LG; >94% for 2-OG; >99% for 1-OG; and >90% for 1-LG. The 2-MG standards were a combination of 90% isomer 2 and 10% isomer 1. The isotopic purity of the MG standards and their deuterated analogs was verified by injecting the individual standard solutions into the LC/MS-MS system with standards and their deuterated analogs was verified by injecting the individual standard solutions into the LC/MS-MS system with standards and their deuterated analogs. The two ISTD mixes of ISTD were spiked into the plasma samples at a fixed volume of 25 μl. The structures of the EC analytes and deuterated analogs are represented in Figs. 1 and 2.
Sample preparation
Freshly extracted blood from human volunteers was collected in 10 ml K2E 18.0 mg (EDTA) BD Vacutainer tubes and centrifuged immediately for 15 min at 2,800 g in a refrigerated centrifuge (4°C). Plasma was then immediately separated from the blood and distributed in aliquots for further processing or stored at −80°C. Discarded human plasma batches from the Blood Bank of Hospital del Mar of Barcelona were used for the validation experiments.

Plasma samples were thawed in less than 30 min at room temperature and processed on ice. Aliquots of 0.5 ml were transferred into glass borosilicate tubes, spiked with 25 μl of ISTD mix

Human volunteers
Three human male volunteers were recruited for the procurement of blood samples following protocol MUESBIOL/1 (protocol for the collection of biological samples for biomedical research studies). Twenty-five female healthy control volunteers with a BMI of <25 kg/m² were recruited for the procurement of blood samples following the TANOBÉ protocol. Both protocols were approved by the Ethical Committee of Parc de Salut Mar Barcelona (CEIC-PSMAR) and comply with the Declaration of Helsinki. An informed consent was obtained from the human subjects.

Fig. 1. Structures of the ECs and ERCs.

Fig. 2. Structures of the deuterated analogs of ECs and ERCs.
The linearity of the method was assessed for seven surrogated analytes (SAs) by construction of calibration curves using plasma samples spiked with deuterated analogs of NAEs and MGs. Analysis was performed in quadruplicate for the following SAs: AEA-d4, LEA-d4, PEA-d4, OEA-d4, DHEA-d4, 2-AG-d5, and 2-OG-d5. The ISTDs were 2-AG-d8 and AEA-d8 (ISTD mix 2), which have additional deuterium atoms in their structure. 2-AG-d8 was used as ISTD of 2-MGs, and AEA-d8 as ISTD of NAEs. The regression analyses of the calibration curves were calculated with SPSS 12.0 with a 1/x weighting factor.

Quantification

Experimental LC/MS-MS parameters for the detection of analytes and the deuterated analogs are presented in Tables 1 and 2. The quantification of the SAs was calculated by interpolation of the response ratios on the calibration curves. The quantification of the authentic analytes was carried out by isotope dilution with the following formula: 

\[
\text{[EC]} \text{ng/ml} = \frac{\text{ng ISTD} \times \text{analyte response}}{\text{ISTD response} \times \text{RF} \times \text{ml aliquot volume}}
\]

The response factor (RF) was calculated as the ratio of the response area of the analyte divided by the response area of its ISTD for a standard solution mix directly injected without extraction into the LC/MS-MS system and in which equal amounts of the analyte and ISTD were injected.

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**TABLE 1.** Experimental LC/MS-MS parameters for the analyte detection

| Analyte | MW | T (min) | CV (%) | P (m/z) | Q (m/z) | I (m/z) | F (V) | CE (eV) | ISTD RF |
|---------|----|---------|--------|---------|---------|---------|-------|---------|---------|
| 2-AG    | 378.6 | 7.65   | 0.08  | 379.2 | 287    | 269, 203 | 135   | 12     | 2-AG-d5 1.00 |
| 1-AG    | 378.6 | 7.77   | 0.30  | 379.2 | 287    | 269, 203 | 135   | 12     | 2-AG-d5 1.00 |
| 2-LG    | 354.5 | 7.76   | 0.06  | 355.2 | 263    | 245, 337 | 135   | 12     | 2-OG-d5 0.79 |
| 1-LG    | 354.5 | 7.94   | 0.08  | 355.2 | 263    | 245, 337 | 135   | 12     | 2-OG-d5 0.79 |
| 2-OG    | 356.5 | 8.61   | 0.41  | 357.3 | 265    | 247, 339 | 135   | 12     | 2-OG-d5 1.00 |
| 1-OG    | 356.5 | 8.82   | 0.08  | 357.3 | 265    | 247, 339 | 135   | 12     | 2-OG-d5 1.00 |
| AEA     | 347.5 | 7.22   | 0.07  | 348.3 | 62     | 44, 287 | 135   | 12     | AEA-d4 1.00 |
| DEA     | 375.6 | 7.99   | 0.08  | 376.3 | 62     | 44      | 135   | 12     | AEA-d4 1.26 |
| DGLEA   | 349.6 | 7.61   | 0.08  | 350.2 | 62     | 44      | 135   | 12     | AEA-d4 1.68 |
| DHEA    | 371.6 | 7.11   | 0.07  | 372.6 | 62     | 44      | 135   | 12     | DHEA-d4 1.00 |
| EPEA    | 345.5 | 6.66   | 0.13  | 346.2 | 62     | 44      | 135   | 12     | AEA-d4 1.00 |
| LEA     | 325.5 | 7.26   | 0.07  | 324.5 | 62     | 44      | 135   | 12     | LEA-d4 1.00 |
| aLEA    | 325.5 | 6.66   | 0.11  | 322.2 | 62     | 44      | 135   | 12     | LEA-d4 1.00 |
| OEA     | 325.5 | 8.05   | 0.06  | 326.1 | 62     | 44, 309 | 135   | 12     | OEA-d4 1.00 |
| PEA     | 299.5 | 7.81   | 0.07  | 300.1 | 62     | 44, 283 | 135   | 12     | PEA-d4 1.00 |
| POEA    | 297.5 | 6.94   | 0.08  | 298.2 | 62     | 44      | 135   | 12     | PEA-d4 1.00 |
| SEA     | 327.5 | 9.11   | 0.07  | 328.1 | 62     | 44, 311 | 135   | 12     | OEA-d4 1.00 |

MW, molecular weight; T, retention time; P, precursor ion; Q, quantifier product ion; I, identifier(s) product ion(s); F, fragmenter; CE, collision energy.

*Response factor of the analyte versus the internal standard.

**TABLE 2.** Experimental LC/MS-MS parameters for the deuterated analogs detection

| Deuterated Analog | MW | T (min) | Q1 (m/z) | Q3 (m/z) | F (V) | CE (eV) |
|------------------|----|---------|----------|----------|-------|---------|
| 2-AG-d5          | 383.6 | 7.63 | 384.3 | 287 | 135 | 12 |
| 1-AG-d5          | 383.6 | 7.77 | 384.3 | 287 | 135 | 12 |
| 2-AG-d8          | 386.6 | 7.59 | 387.5 | 295 | 135 | 12 |
| 2-OG-d5          | 361.6 | 8.59 | 362.2 | 265 | 135 | 12 |
| 1-OG-d5          | 361.6 | 8.79 | 362.2 | 265 | 135 | 12 |
| AEA-d4           | 351.6 | 7.21 | 352.2 | 265 | 135 | 12 |
| AEA-d8           | 355.6 | 7.19 | 356.2 | 62 | 135 | 12 |
| DHEA-d4          | 375.6 | 7.09 | 376.2 | 66 | 135 | 12 |
| LEA-d4           | 327.5 | 7.24 | 328.5 | 66 | 135 | 12 |
| OEA-d4           | 329.6 | 8.04 | 330.4 | 66 | 135 | 12 |
| PEA-d4           | 303.5 | 7.79 | 304.4 | 66 | 135 | 12 |

MW, molecular weight; T, retention time; Q1, precursor ion; Q3, product ion; F, fragmenter; CE, collision energy.
and ISTD were present. A deuterated form was not commercially available for some analytes, so a deuterated analog of another NAE or MG with a similar structure was used as ISTD. ISTD mix 1 was used for the quantification of authentic analytes. This fit-for-purpose approach could be employed due to the fact that the basic structure of the NAEs and the MGs is the same, the only difference being the length of the hydrocarbon chain and the number and position of double bonds. For some analytes the RF was considered 1.0 because the differences in the absolute response were less than 10% (Table 1). We found that the responses of 2-AG-d8 and AEA-d8 were considerably lower than their non-deuterated forms (approximately 10-fold), although, as they were not used in the isotope dilution quantification method, calculations were not affected. The decreased response was probably due to the different position of the deuterium atoms in the structure of the d8 analog (and next to the double bonds) compared with the d4 and d5 analogs (Fig. 2). In our LC/MS-MS conditions, responses of the 1-MG and 2-MG isomers were the same.

Limits of detection and quantification

The mathematical estimates of the limits of detection (LODs) and lower limits of quantification (LLOQs) of the SAs were inferred from the equations of the curves by the following formulas: LOD ng/ml = (SD of the replicates of the lowest concentration on calibrator/slope) × 3 and LLOQ ng/ml = (SD of the replicates of the lowest concentration on calibrator/slope) × 10. Additionally, the LLOQs of the SAs were verified experimentally by a six replicate analyses of plasma spiked with d4 or d5 deuterated forms of NAEs and 2-MGs at the following concentrations: PEA-d4, 0.1 ng/ml; OEA-d4, 0.1 ng/ml; LEA-d4, 0.1 ng/ml; AEA-d4, 0.02 ng/ml; DHEA-d4, 0.02 ng/ml; 2-AG-d5, 0.75 ng/ml; and 2-OG-d5, 2.5 ng/ml. The samples were further spiked with ISTD mix 2, which contained 2-AG-d8 and AEA-d8, and were analyzed by LC/MS-MS. The ratio of the SAs and their ISTDs was calculated. A coefficient of variation (CV) of the ratios of less than 20% and a signal to noise ratio greater than three were considered acceptable.

A dilution integrity experiment was carried out for lower sample volumes down to 50 μl, with no significant differences in concentration. However, the standard volume of the method was set at 0.5 ml in order to be able to quantify the ECs and ERCs with lower endogenous concentrations.

Accuracy and imprecision

The within-day and between-day accuracy and imprecision of the method were evaluated by the quadruplicate analysis of quality control (QC) samples at three concentration levels [QC-low (L), QC-mid (M), and QC-high (H)] over a 3 day validation protocol. The QC samples were prepared by spiking a batch of plasma on top of its basal EC and ERC concentrations. QC-L was spiked at 0.05 ng/ml POEA, DGLEA, EPEA, ALA, LEA, AEA, DEA, and DHEA; 1 ng/ml 2-AG, PEA, OEA, and SEA; and 5 ng/ml 2-OG and 2-LG. QC-M was spiked at 0.5 ng/ml POEA, DGLEA, EPEA, LEA, N-α-linolenoyl ethanolamide (α-LEA), AEA, DEA, and DHEA; 5 ng/ml 2-AG, PEA, OEA, and SEA; and 50 ng/ml of 2-OG and 2-LG. QC-H was spiked at 2.5 ng/ml POEA, DGLEA, EPEA, LEA, α-LEA, AEA, DEA, and DHEA; 25 ng/ml 2-AG, PEA, OEA, and SEA; and 250 ng/ml 2-OG and 2-LG. Aliquots of each QC were distributed into cryotubes and stored at −80°C until analysis. The samples were randomly analyzed in order to assess carry over.

Aliquots of 0.5 ml of the QC samples were spiked with ISTD mix 1 at the following amounts of deuterated analogs: 0.25 ng AEA-d4, 0.25 ng DHEA-d4, 0.50 ng LEA-d4, 1 ng PEA-d4, 1 ng OEA-d4, 5 ng 2-AG-d5, and 25 ng 2-OG-d5; and analyzed by LC/MS-MS. The quantification was done by isotope dilution.

### TABLE 3. Stability of the isomerization upon extraction from the biological matrix

| Extraction Solvent       | 2-AG-d5 (ISO1/ISO2) | 2-OG-d5 (ISO1/ISO2) |
|--------------------------|---------------------|---------------------|
| Spiked plasma            |                     |                     |
| TBME:water (6:1, v/v)    | 0.59                | 0.32                |
| TBME:water (6:1, v/v)    | 0.53                | 0.20                |
| TBME:Ac. 0.1 M, pH 4.0 (6:1, v/v) | 0.10 | 0.09                |
| Toluene:water (6:1, v/v) | 1.40                | 0.47                |
| Standard solution        | 0.06                | 0.05                |

The stability of the isomerization of isomer 2 to isomer 1 during extraction and evaporation steps was assessed by analyzing the ratio (ISO1/ISO2) of plasma spiked with standard solutions of 2-AG-d5 and 2-OG-d5 subjected to liquid-liquid extraction compared with standard solutions dissolved in mobile phase injected directly into the LC/MS-MS system. ISO1, isomer 1; ISO2, isomer 2.

Accuracy was calculated as the percentage of difference between the observed concentration and the nominal concentration. The nominal concentration was calculated as the expected concentration on day 1 of the QC sample after the spiking process, taking into account the basal EC and ERC concentrations. A percentage of difference less than 15% for QC-M and QC-H, and less than 20% for QC-L was considered acceptable.

Imprecision was calculated as the standard error deviation of the QC sample replicates. A standard error deviation less than 15% for QC-M and QC-H, and less than 20% for QC-L was considered acceptable.

Recovery and matrix effect

Recovery and matrix effect were evaluated in plasma from six different sources with deuterated analogs as SAs analyzed in triplicate. First, each batch of plasma was divided into two pools; one pool was spiked with ISTD mix 1 and mix 2 and extracted, while the other pool was spiked with ISTD mix 1 and mix 2 after extraction. Second, ISTD mix 1 and mix 2 were also spiked into clean glass tubes, evaporated, and reconstituted. Finally, extracted samples and pure standards were analyzed by LC/MS-MS. Recovery was calculated as the response of the SAs of samples spiked before extraction versus samples spiked after extraction. The matrix effect was calculated as the response of the SAs of samples spiked after extraction versus the pure standards. Additionally, the CV of the ratio of the SAs with the ISTD of the six plasma sources was calculated.

Stability of the analytes on reinjection

The stability of the reconstituted extract solutions on HPLC vials was tested with the reinjection of a batch in which the vials were kept at 4°C for 24 h and another batch with the vials kept at −20°C for 10 days.

Stability of the isomers 1 and 2 of MGs in the biological matrix

The stability of the isomers 1 and 2 of MGs to isomerization (or acyl migration) in the biological matrix was assessed by measuring the ratio between the isomers after preservation of the plasma at different times and temperatures. For that, one batch of human plasma from the blood bank was spiked with 2-OG-d5 and 2-AG-d5 and another batch was spiked with 1-OG-d5 and 1-AG-d5. Samples were analyzed by LC/MS-MS and the ratios of the 1 and 2 isomers of both batches were calculated. The spiked standards, dissolved in mobile phase, were also injected directly into the LC/MS-MS system and the
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KT109, and GSK 264220A. Stock inhibitor solutions were prepared in ethanol, except D609 that was prepared in water. The volume of spiking solution added was less than 2% with respect to the plasma aliquot volume. Plasma was distributed into cryotubes for the incubation experiments. Edelfosine is a phosphatidylinositol phospholipase C inhibitor (IC50 = 9.6 μM). D609 is a phosphatidylcholine-specific phospholipase C inhibitor (K = 6.4 μM). FIPI is a phospholipase D1 and D3 inhibitor (IC50 = 20 and 25 nM, respectively). Orlistat is a gastric and pancreatic lipase inhibitor and a nonselective DAGLα and DAGLβ inhibitor (IC50 = 60 and 100 nM, respectively). RHC 80267 is a nonselective DAGL inhibitor (IC50 = 4 μM). KT172 and KT109 are selective DAGLβ inhibitors (IC50 = 60 and 42 nM, respectively) and also DAGLα inhibitors (IC50 = 0.14 and 2.3 μM, respectively). GSK 264220A is an endothelial lipase and a lipoprotein lipase inhibitor (IC50 = 0.13 and 0.10 μM, respectively). The effect of the blood collection tube on the MG production was assessed on EDTA (K2E, 18 mg) or Lithium heparin (LH, 170 IU) in 10 ml BD Vacutainer tubes with or without the presence of the inhibitor. All the inhibitors were tested with EDTA-plasma while Orlistat and GSK 264220A were additionally tested on heparin-plasma. The estimation of the IC50 of Orlistat for the generation of 2-AG, 2-LG, and 2-OG was done in EDTA-plasma samples from three human volunteers with the following added concentrations of Orlistat: 0, 50, 150, 450, 900, 1,500, and 2,500 nM. Control plasma samples were kept at 4°C for 2 h until analysis. The percentage of inhibition was calculated with respect to the levels at concentration of inhibitor 0 of each plasma source. The data were modeled by the software GraphPad Prism 5 with the inhibition model: log [inhibitor] versus percent inhibition and the IC50s for 2-AG, 2-LG, and 2-OG were calculated.

Stabilization of MG measures in plasma with Orlistat

Blood extracted from 25 human female volunteers was collected in 10 ml K2E 18.0 mg (EDTA) BD Vacutainer tubes and centrifuged immediately at 2,800 g in a refrigerated centrifuge (4°C). Plasma of each volunteer was separated immediately from the blood and two equal 0.6 ml aliquots were obtained. One aliquot was spiked at 3.35 μM with 5 μl of Orlistat solution (200 g/ml, ethanol). Both aliquots were stored at −80°C until EC analysis with our standard procedure.

RESULTS AND DISCUSSION

Method development

While some solid phase extraction methods were tried, recovery was difficult to optimize due to the varying structures of the analyzed compounds (NAEs and MGs). The

Table 4. Recovery and matrix interference of the SAs in plasma

| Surrogate Analyte | ISTD   | Recovery (%) | Matrix Effect (%) | CV (%) |
|-------------------|--------|--------------|------------------|--------|
| PEA-d4            | OEA-d4 | 96 ± 11      | −26 ± 4.7        | 3.0    |
| LEA-d4            | OEA-d4 | 96 ± 7.1     | −19 ± 4.8        | 3.0    |
| OEA-d4            | PEA-d4 | 95 ± 8.4     | −19 ± 5.8        | 3.4    |
| AEA-d4            | AEA-d8 | 95 ± 7.9     | −15 ± 4.9        | 3.5    |
| DHEA-d4           | AEA-d4 | 89 ± 7.6     | −11 ± 6.0        | 2.9    |
| AEA-d8            | AEA-d4 | 84 ± 8.7     | −8.0 ± 5.0       | 4.2    |
| 2-OG-d5           | 2-AG-d5| 84 ± 8.0     | −40 ± 5.8        | 7.9    |
| 2-AG-d5           | 2-AG-d8| 85 ± 7.8     | −27 ± 4.0        | 3.9    |
| 2-AG-d8           | 2-AG-d5| 81 ± 6.8     | −16 ± 4.3        | 4.9    |

Mean ± SD of the recovery and matrix effect of the surrogate analytes in plasma of six different sources and analyzed in triplicate; CV of the ratio of the SAs with the ISTDS of the six plasma sources.
liquid-liquid extraction methods provided the best overall recoveries for ECs and ERCs. In order to assess a method to stabilize the original isomeric ratio after the extraction and evaporation steps, 0.5 ml plasma samples were spiked with the deuterated analog isomers 2-AG-d5 and 2-OG-d5, extracted, and analyzed by LC/MS-MS to evaluate the generation of 1-AG-d5 and 1-OG-d5 by chemical isomerization or acyl migration (Table 3). Several liquid-liquid extraction methods were tested: TBME or toluene as extraction solvents, Am. Ac. buffer 0.1 M at pH 4.0, or water as aqueous solvents, and new silanized borosilicate glass tubes (or clean reused ones, or new silanized tubes).

We found optimal recoveries and peak shapes with the reconstitution of the extract in a mixture of water: acetonitrile (10:90) with 0.1% formic acid. The EC and ERC profiles were separated by reverse phase gradient chromatography in a C18 column (Fig. 3), because with a C8 column complete separation of the MG isomers was not possible. Acetonitrile was used as the organic mobile phase, as we observed that methanol also promoted 2-MG/1-MG isomerization if present in the mobile phase. Formic acid at 0.1% v/v was employed as an additive of the mobile phase to promote the positive ionization of NAEs and MGs. The parent ion adducts selected for fragmentation in the mass spectrometer were in the form of [M+H]+.

The product ion m/z 62, which corresponds to ethanolamine, is characteristically generated by fragmentation of NAEs; while a neutral loss of 92 Da, which corresponds to

| Analyte | QC-L | QC-M | QC-H | QC-L | QC-M | QC-H | QC-L | QC-M | QC-H | QC-L | QC-M | QC-H |
|---------|------|------|------|------|------|------|------|------|------|------|------|------|
| 2-AG    | 8.7  | 9.0  | 7.2  | 88.7 | 91.0 | 75.6 | 11.0 | 18.4 | 14.8 | 88.4 | 71.7 | 83.3 |
| 2/1-AG  | 6.7  | 5.7  | 5.7  | 96.9 | 96.4 | 93.5 | 7.4  | 14.3 | 8.2  | 88.7 | 93.2 | 102.8 |
| 24-LG   | 11.7 | 12.8 | 5.9  | 95.8 | 91.3 | 77.2 | 16.2 | 18.2 | 19.5 | 80.2 | 76.3 | 83.2 |
| 2/1-LG  | 5.4  | 8.5  | 5.7  | 94.9 | 100.6| 104.7| 11.2 | 4.8  | 9.6  | 91.4 | 101.2| 109.6 |
| 24OG    | 10.6 | 7.6  | 8.6  | 86.9 | 92.9 | 83.2 | 10.6 | 18.3 | 8.2  | 88.4 | 77.5 | 89.5 |
| 2/1-OG  | 9.7  | 8.7  | 6.9  | 95.3 | 89.9 | 97.0 | 12.1 | 11.2 | 5.6  | 90.8 | 85.1 | 104.4 |
| LEA     | 4.6  | 6.1  | 4.3  | 92.9 | 88.7 | 89.6 | 9.8  | 10.2 | 4.8  | 92.1 | 92.9 | 89.4 |
| DEA     | 4.5  | 6.1  | 5.2  | 91.6 | 91.4 | 93.0 | 11.9 | 6.9  | 5.3  | 95.0 | 94.1 | 95.2 |
| DGLEA   | 7.7  | 6.1  | 4.1  | 97.0 | 97.6 | 95.0 | 10.4 | 9.0  | 5.4  | 101.2| 97.5 | 98.3 |
| DHEA    | 4.7  | 8.5  | 5.0  | 92.1 | 90.0 | 92.1 | 10.2 | 9.3  | 4.6  | 90.9 | 94.5 | 93.7 |
| EPEA    | 7.2  | 5.3  | 4.3  | 95.3 | 89.9 | 88.0 | 9.8  | 10.0 | 6.1  | 90.6 | 89.1 | 89.7 |
| LEA     | 3.2  | 7.4  | 5.4  | 97.8 | 97.9 | 97.5 | 10.0 | 10.2 | 5.5  | 94.8 | 99.9 | 101.1 |
| α-LEA   | 5.6  | 9.0  | 5.7  | 93.9 | 88.5 | 87.0 | 11.5 | 12.5 | 8.1  | 86.7 | 88.5 | 88.1 |
| OEA     | 2.9  | 7.5  | 3.8  | 97.3 | 98.4 | 96.4 | 9.3  | 9.7  | 4.6  | 94.9 | 97.2 | 101.6 |
| PEA     | 2.9  | 6.6  | 3.0  | 95.4 | 97.6 | 96.7 | 9.4  | 9.5  | 3.9  | 94.5 | 96.5 | 100.2 |
| POEA    | 7.0  | 11.3 | 6.2  | 96.5 | 93.8 | 86.3 | 14.1 | 12.4 | 9.8  | 92.9 | 89.7 | 89.8 |
| SEA     | 4.9  | 9.4  | 3.8  | 93.3 | 98.3 | 99.0 | 10.8 | 10.5 | 8.8  | 100.8| 97.2 | 104.2 |

Data represent the mean values of QC sample replicates.

Table 3. Imprecision and accuracy

| Aluminium | Accuracy (%) | Accuracy (%) | Accuracy (%) | Accuracy (%) |
|-----------|--------------|--------------|--------------|--------------|
| 2/1-AG    | QC-L         | QC-M         | QC-H         | QC-L         |
| 24-LG     | 6.7          | 5.7          | 5.7          | 96.9         |
| 2/1-LG    | 5.4          | 8.5          | 5.7          | 94.9         |
| 24OG      | 10.6         | 7.6          | 8.6          | 86.9         |
| 2/1-OG    | 9.7          | 8.7          | 6.9          | 95.3         |
| LEA       | 4.6          | 6.1          | 4.3          | 92.9         |
| DEA       | 4.5          | 6.1          | 5.2          | 91.6         |
| DGLEA     | 7.7          | 6.1          | 4.1          | 97.0         |
| DHEA      | 4.7          | 8.5          | 5.0          | 92.1         |
| EPEA      | 7.2          | 5.3          | 4.3          | 95.3         |
| LEA       | 3.2          | 7.4          | 5.4          | 97.8         |
| α-LEA     | 5.6          | 9.0          | 5.7          | 93.9         |
| OEA       | 2.9          | 7.5          | 3.8          | 97.3         |
| PEA       | 2.9          | 6.6          | 3.0          | 95.4         |
| POEA      | 7.0          | 11.3         | 6.2          | 96.5         |
| SEA       | 4.9          | 9.4          | 3.8          | 93.3         |

Table 4. Stability of MG concentrations on different collection conditions

| Blood Tube | Inhibitor in Plasma | 2/1-AG | 2/1-OG |
|------------|---------------------|--------|--------|
|            |                     | Time 0 | Time 0 |
| EDTA       | −                   | 0.75 ± 0.21 | 8.51 ± 4.57 |
| Heparin    | −                   | 2.82 ± 0.67 | 30.6 ± 23.0 |
| Heparin    | +                   | 2.99 ± 0.05 | 65.3 ± 3.77 |

Blood was collected in EDTA or heparin tubes. The separated plasma was analyzed immediately (time 0) or after incubation for 2 h at room temperature with or without addition of Orlistat to the plasma collection tube. Orlistat was added at 3.5 μM to EDTA-plasma and at 15 μM to heparin-plasma. Data are presented as mean ± SD of 2/1-MG concentrations (ng/mL) of plasma from one volunteer in an experiment performed in duplicate.
The quantification of ECs, as for all endogenous analytes, is challenging due to the absence of a blank matrix. Some authors have developed strategies of depletion of the analytes by processing the plasma using five cycles of activated charcoal (12). Other authors have used a surrogated analysis approach (18) or carried out quantification by isotope dilution (19, 20). It is to be noted that EC-depleted plasma still contains MGs, probably due to its high concentrations (12). In this work, we have assessed the linearity, LOD, LLOQ, recovery, matrix effect, and MG acyl migration stability of the method using deuterated analog forms as SAs and ISTDs. Our approach is valid because the original unaltered matrix can be used and, theoretically, the deuterated forms have the same properties as the authentic analytes. However, there are a limited number of deuterated analogs of ECs and ERCs, and in order to use this approach different deuterated analog versions for each analyte are necessary. For this reason, in the analysis of samples of clinical studies (inhibition experiments), and in determining the accuracy and imprecision of the method, quantification was carried out with isotope dilution as described in the Materials and Methods.

Method validation

The method was linear for the ECs and ERCs whose quantification was standardized. Results, which include the mathematically derived LODs and LLOQs, are shown in supplementary Table I. The experimentally verified LLOQs of the method are the following: 0.02 ng/ml for AEA, DEA, DGLEA, EPEA, α-LEA, DHEA and POEA; 0.1 ng/ml for LEA; 0.5 ng/ml for OEA and SEA; 0.75 ng/ml for 2-AG; 1 ng/ml for PEA; and 2.5 ng/ml for 2-OG and 2-LG. The LLOQs of PEA, OEA, and SEA were set at a higher concentration than their mathematical LLOQs due to small basal contaminant concentrations found in the solvents and glassware as reported by other authors (12, 21). No significant carry over was detected. No differences in the concentration values were found after reinjecting vials kept at 4°C for 24 h. Vials kept at 20°C were stable for all analytes except SEA. Recoveries were high (>80%) for all the analytes and matrix effect was substantial (40%) in some analytes such as 2-OG-d5. Minimal differences in the matrix effect of the six plasma sources were, however, observed due to being compensated by the use of deuterated analogs with similar ISTD structure (Table 4).

Within-run and between-run accuracy and imprecision values of NAEs and MGs are presented in Table 5 and fit current standard requirements for analytical method validation. With respect to the MGs, we found decreases in the concentration of the separate 2-MG isomers from day 2 of the validation protocol due to acyl migration during conservation of the plasma. This is explained in the section on stability of the isomeric ratio of MGs. The method is deemed fit for the determination of the EC and ERC profile in human plasma samples.

Inhibition experiments of the ex vivo generation of MGs from plasma

Fanelli et al. (12) reported the generation of 2-AG in plasma in the absence of blood cells, with increases in 2/1-AG for plasma preserved for 4 h at 4°C or room temperature. In the course of our MG stability experiments, we observed
and IC 50 >15

pounds showed inhibition activity (IC 50 >30

specific inhibitor of DAGL
tested the inhibition activity of RHC 80267, another non-
activity. Because Orlistat is a general lipase inhibitor, we also
this MG buildup should be related to an enzymatic plasma
that essentially eliminates all blood cells, which means that
of MG persisted even after immediate sample centrifugation
DAGL (22) and a potent nonspecific inhibitor of DAGL
spiked with Orlistat, a gastric and pancreatic lipase inhibitor
the ex vivo generation of 2-AG, 2-LG, and 2-OG in plasma
respectively). On the other hand, we observed inhibition of
the precursors of 2-AG, are originated by the hydrolysis of
membrane phosphoinositides and they are converted to
2-AG by the action of two sn-1 selective DAGLs, DAGLα and
DAGLβ (16). Further, a direct dietary origin of 2-AG seems
unlikely because arachidonic acid, an essential fatty acid
and backbone of 2-AG structure, is present at low amounts
in the diet, and is mainly obtained through metabolism of
triacylglycerols that contain acyl-linoleoyl in their structure.
The linoleic acid released is then elongated and unsaturated to form arachidonic acid through the omega-6
pathway. Additionally, we investigated the effect of the
blood collection tube on MG generation. We found that
MG concentrations were higher in heparin-plasma than in
EDTA-plasma. The differences were maintained either for
samples analyzed immediately or after incubation at room
of DAGL. 2-OG and 2-LG originate from fat digestion in
the intestinal lumen, where dietary triacylglycerol is hydrolyzed in the sn-1 and sn-3 position by pancreatic lipase
through a series of directed stepwise reactions to diacylglycerol (DAG), 2-MG, fatty acids, and glycerol. 2-MGs are readily adsorbed and resynthesized to triacylglycerols through the MG pathway (7, 25–27). Therefore, in terms of preventing 2-MG ex vivo formation, results obtained from plasma samples spiked with Orlistat are in agreement with this inhibitory enzymatic activity. The biosynthetic origin of 2-AG, however, is presumably not related to fat digestion but to phospholipids. Arachidonate DAGs, the precursors of 2-AG, are originated by the hydrolysis of membrane phosphoinositides and they are converted to 2-AG by the action of two sn-1 selective DAGLs, DAGLα and DAGLβ (16). Further, a direct dietary origin of 2-AG seems unlikely because arachidonic acid, an essential fatty acid and backbone of 2-AG structure, is present at low amounts in the diet, and is mainly obtained through metabolism of triacylglycerols that contain acyl-linoleoyl in their structure. The linoleic acid released is then elongated and unsaturated to form arachidonic acid through the omega-6

| Measured Concentration (ng/ml) |
|-------------------------------|
| EC/ERC | n | Without Added Orlistat | With Added Orlistat | Change (%) | P |
| 2/1-AG | 25 | 2.42 ± 1.10 | 0.89 ± 0.50 | −50.0 ± 23.9 | <0.001 |
| 2/1-LG | 25 | 15.2 ± 8.48 | 7.76 ± 3.54 | −47.2 ± 17.5 | <0.001 |
| 2/1-OG | 25 | 16.1 ± 10.4 | 8.93 ± 4.90 | −35.9 ± 25.0 | <0.001 |
| AEA | 25 | 0.40 ± 0.19 | 0.39 ± 0.19 | −2.90 ± 9.58 | 0.225 |
| DEA | 25 | 0.12 ± 0.04 | 0.11 ± 0.04 | −0.45 ± 11.8 | 0.671 |
| DGLEA | 25 | 0.11 ± 0.03 | 0.12 ± 0.03 | −0.33 ± 12.3 | 0.324 |
| DHEA | 25 | 0.45 ± 0.19 | 0.44 ± 0.21 | −0.74 ± 10.9 | 0.801 |
| EPEA | 14 | 0.05 ± 0.01 | 0.05 ± 0.01 | −8.67 ± 9.02 | 0.104 |
| LEA | 25 | 1.30 ± 0.38 | 1.25 ± 0.38 | −4.05 ± 11.7 | 0.091 |
| α-LEA | 25 | 0.04 ± 0.01 | 0.04 ± 0.01 | −4.39 ± 13.4 | 0.142 |
| OEA | 25 | 3.16 ± 1.26 | 3.10 ± 1.22 | −1.72 ± 5.46 | 0.057 |
| PEA | 25 | 2.07 ± 0.67 | 2.06 ± 0.60 | 0.75 ± 9.20 | 0.774 |
| POEA | 25 | 0.18 ± 0.11 | 0.17 ± 0.10 | −2.90 ± 12.0 | 0.491 |
| SEA | 25 | 1.18 ± 0.33 | 1.15 ± 0.32 | −2.15 ± 8.81 | 0.214 |

Plasma of 25 female human volunteers was collected with or without addition of Orlistat (3.4 µM) and stored at −80°C until EC analysis. Data are presented as mean ± SD. EPEA concentration was below the LLOQs for some of the samples. The effect of Orlistat addition to plasma on EC measures was assessed by a paired-samples t-test.

TABLE 8. Stability of the isomerization of spiked 2/1-AG-d5 and 2/1-OG-d5 in stored plasma

| Spiked analyte | Time 0 | Time, 30 min at room temperature | Time, 20 days at −20°C | Time, 20 days at −80°C |
|----------------|--------|---------------------------------|------------------------|------------------------|
| 2/1-AG | ISO1/ISO2 | 0.08 ± 0.001 | 0.46 ± 0.06 | 0.84 ± 0.05 | 0.41 ± 0.01 |
| 1-AG-d5 | ISO2/ISO1 | 0.01 ± 0.01 | 0.04 ± 0.005 | 0.07 ± 0.003 | 0.03 ± 0.002 |
| 2-OG | ISO1/ISO2 | 0.08 ± 0.01 | 0.56 ± 0.06 | 0.58 ± 0.03 | 0.36 ± 0.06 |
| 2-OG-d5 | ISO2/ISO1 | 0.13 ± 0.03 | 0.13 ± 0.03 | 0.12 ± 0.04 | 0.13 ± 0.03 |

A pool of plasma was spiked separately with the deuterated analogs of the isomer 1 (ISO1) and isomer 2 (ISO2) of AG and OG. Aliquots were distributed in cryotubes and chemical stability of the isomer ratio (ISO1/ISO2 and ISO2/ISO1) was assessed upon conservation at time 0, 30 min at room temperature, 20 days at −20°C, or 20 days at −80°C. Data are presented as mean ± SD of replicate analysis.
endogenous analyte Time 2 h at 4°C Time 2 h at room temperature

| Endogenous analyte | Ratio ISO1 / ISO2 |
|--------------------|------------------|
| 2/1-AG             | 0.09 ± 0.10      |
|                    | 0.32 ± 0.24      |
| 2/1-LG             | 0.43 ± 0.13      |
|                    | 0.76 ± 0.10      |
| 2/1-OG             | 0.28 ± 0.08      |
|                    | 0.57 ± 0.06      |

The stability of the isomer 1/isomer 2 (ISO1/ISO2) ratio of endogenous 2/1-AG, 2/1-LG, and 2/1-OG was assessed in plasma of three different volunteers which was kept 2 h at 4°C or room temperature after extraction from the volunteer. Data are presented as mean ± SD of triplicate analyses.

temperature. Orlistat was able to inhibit MG production in plasma originated from both kind of tubes, but due to the higher MG buildup in heparin-plasma, a higher concentration of Orlistat was needed to achieve full inhibition (Table 6). Further, because heparin-plasma is commonly used for the assay of lipoprotein lipase due to its affinity for heparin (28), we also tested the inhibition activity of GSK 264220A, an endothelial lipase and a lipoprotein lipase inhibitor (29), but we found no inhibition (IC$_{50}$ > 15 μM) on EDTA-plasma or heparin-plasma. The reason for the lower MG concentrations on EDTA-plasma is probably due to the chelate effect of EDTA on the cofactors needed for MG biosynthesis. We recommend, therefore, the use of EDTA blood tubes for collection in addition to Orlistat. In a second set of experiments, EDTA-plasma of three human volunteers was used for the calculation of the IC$_{50}$ of Orlistat for the ex vivo generation of 2-AG, 2-LG, and 2-OG. An inhibition model was obtained and the data is graphically presented in Fig. 4. The IC$_{50}$ of Orlistat with the mean, its 95% confidence interval, and the coefficient of determination (R$^2$) of the inhibition model are as follows: 285.6 nM [212.4, 384.0] for 2-AG (R$^2$ = 0.8809), 146.1 nM [104.9, 203.4] for 2-LG (R$^2$ = 0.9087), and 148.7 nM [110.6, 200.0] for 2-OG (R$^2$ = 0.9254).

**Stabilization of MG measures in plasma spiked with Orlistat**

The ex vivo generation of MG in plasma can be prevented by Orlistat. Because our MG assay was done at room temperature, we tested, in controlled conditions, whether Orlistat addition to the plasma storage tube had any effect on MG measures when a typical clinical sample collection protocol was followed. For that, 25 female blood samples were collected and processed in a matter of weeks, maintaining the cold chain until they were finally stored at −80°C. EC analysis took place several weeks after all samples had been collected and was done with our standard sample preparation procedure. The results are presented in Table 7 and they show that Orlistat addition during the sample collection protocol leads to a significant reduction of all MG measures (36–59%, P < 0.001). The NAEs, which are the other measures of our EC and ERC analysis, were not affected by the addition of Orlistat. We think that differences may have arisen due to the enzymatic activity that took in the freezing/thawing and processing steps. All the NAEs and MGs described in the method could be quantified in the 25 human female samples (Table 7), with the exception of EPEA that, due to its low levels, could only be quantified in 14 samples. In summary, data show that Orlistat addition as part of the sample collection protocol can be a tool to stabilize MG concentrations in plasma, and this can aid in the harmonization of EC and ERC measurements in clinical samples.

**Stability of the isomeric ratio of MGs**

The results of the stability experiment of MGs in plasma show that the chemical isomerization of isomer 2 to isomer 1 decreases with decreases in the preservation temperature of the plasma before analysis. However, isomerization is still observed, even if samples are stored at −80°C (Table 8). Furthermore, it has been reported that isomerization is also dependent on the amount of serum albumin present in the sample (17). It is, therefore, possible that even when samples are subjected to the same storage conditions, they could still have different chemical isomerization rates. Additionally, chemical isomerization takes place in plasma preserved for a very short time at room temperature.

We also investigated the endogenous origin of MG isomers in fresh plasma samples (Table 9). As has been previously suggested (14), our data support the hypothesis that 1-AG does not have an endogenous origin and is the result of chemical isomerization during sample storage and processing, because in fresh plasma samples we found that 1-AG was present at the same isomeric ratio as the pure standard mixture. On the other hand, we observed that 1-LG and 1-OG were present at substantial concentrations. 1-MG originates from the in vivo isomerization of 2-MG during digestion and absorption. It has been estimated that approximately 25% of 2-MG is isomerized to the 1-MG form. However, 2-MG is the predominant form in which MGs are absorbed and resynthesized to triacylglycerols, while 1-MGs are eventually hydrolyzed by pancreatic lipase to free fatty acids and glycerol (25–27, 30).

In summary, only EC analysis performed with fresh samples is able to quantify the original isomeric ratio of the sample. Studies that report MG concentrations should specify whether the concentration data are from the separate or combined 1 and 2 isomers. Due to the instability of isomerization during conservation, and the fact that the 1-MG isomer originates either in vivo or ex vivo from the 2-MG isomer, studies that report the concentration of the two isomers together may still provide meaningful data for the interpretation of its biological significance in a fit-for-purpose approach. Alternatively, clinical samples may be spiked with deuterated analogs of known isomer ratios before conservation in order to correct the concentration data.

**CONCLUSIONS**

MG analysis is a challenging issue; to our knowledge, this is the first time that an enzymatic activity inhibited by the lipase inhibitor Orlistat and able to generate MGs in plasma in the absence of cells has been reported. Our findings suggest that, as happens with EC brain concentrations (31),

**TABLE 9. Stability of the isomerization of endogenous 2/1-AG, 2/1-LG, and 2/1-OG in stored plasma**

| Endogenous analyte | Ratio ISO1 / ISO2 |
|--------------------|------------------|
| 2/1-AG             | 0.09 ± 0.10      |
|                    | 0.32 ± 0.24      |
| 2/1-LG             | 0.43 ± 0.13      |
|                    | 0.76 ± 0.10      |
| 2/1-OG             | 0.28 ± 0.08      |
|                    | 0.57 ± 0.06      |

The stability of the isomer 1/isomer 2 (ISO1/ISO2) ratio of endogenous 2/1-AG, 2/1-LG, and 2/1-OG was assessed in plasma of three different volunteers which was kept 2 h at 4°C or room temperature after extraction from the volunteer. Data are presented as mean ± SD of triplicate analyses.
peripheral EC concentrations from clinical studies or animal models greatly depend on sample collection and sample-time processing conditions that take place in the clinical and laboratory settings due to the natural presence of enzymatic activity in plasma. Because of the instability of EC concentrations in blood, EC studies need to follow strict harmonized sample collection and processing protocols in order to avoid artificial differences between samples. Finally, the collection of plasma samples with Orlistat may be a useful tool in the determination of real endogenous 2-MG concentrations. In addition to immediate centrifugation in refrigerated conditions and separation of plasma from blood to avoid the release of NAE from blood cells, we also recommend the addition of Orlistat to plasma collecting tubes and maintaining the cold chain until storage and processing. Orlistat is inexpensive, and thus may be a cost-effective measure to aid in the harmonization of EC and ERC measurements in clinical research. Data suggest that the ex vivo generation of MG in plasma is a mechanism independent of DAGL, because besides the general lipase inhibitor Orlistat, other specific or unspecific DAGL inhibitors do not inhibit MG generation, and neither is the result of endothelial lipase or lipoprotein lipase activity. The full characterization of this enzymatic activity goes beyond the scope of this work, but due to the importance of the EC 2-AG as a bioactive signal, we added here a preliminary description of the enzymatic activity involved in the generation of EC 2-AG from endogenous and exogenous DAGs.

The rate of enzymes like lipases and lipases like lipoprotein lipase is important to study because this enzyme is known to degrade 2-arachidonoyl-glycerol cannabinoid activity. J. Biol. Chem. 286: 31312–31314.

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