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

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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-acylethanolamides 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: artifactual 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-acylethanolamides (NAEs) such as N-linoleoyl ethanolamide (LEA), N-oleoyl ethanolamide (OEA), N-palmitoyl ethanolamide (PEA), N-palmitoleoyl ethanolamide

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Abbreviations: AEA, N-arachidonoyl ethanolamide; AG, arachidonoylglycerol; Am. Ac., ammonium acetate; CB, cannabinoid; CV, coefficient of variation; DAG, diacylglycerol; DAGL, *sn*-1-diacylglycerol lipase; DEA, N-docosatetraenoyl ethanolamide; DGLEA, N-dihomo-γ-linolenoyl ethanolamide; DHEA, N-docosahexaenoyl ethanolamide; EC, endocannabinoid; EPEA, N-eicosapentaenoyl ethanolamide; ERC, endocannabinoid-related compound; ISTD, internal standard; LEA, N-linoleoyl ethanolamide; α-LEA, N-α-linolenoyl ethanolamide; LG, linoleoylglycerol; LLOQ, lower limit of quantification; LOD, limit of detection; MG, monoacylglycerol; MRM, multiple reaction monitoring; NAE, N-acylethanolamide; OEA, 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, surrogated analyte; SEA, N-stearoyl ethanolamide; TBME, tert-butyl-methyl-ether.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of one table.

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(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) (6) 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-α (OEA) (9). Finally, two putative ECs, N-eicosapentaenoyl ethanolamide (EPEA) 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 glycerol. 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-hydrolase-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 phosphatidylinositols, 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 Harbor, 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 939055-18-2), 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 isomeric purity of the MG standards and their deuterated analogs was verified by injecting the individual standard solutions into the LC/MS-MS system with the following results: 91.2%, 2-AG; 91.5%, 2-AG-d5; 100%, 1-AG; 100%, 1-AG-d5; 97.3%, 2-OG; 94.3%, 2-OG-d5; 100%, 1-OG; 88.0%, 1-OG-d5; 98.7%, 2-LG; and 100%, 1-LG. The isotopic purity of the deuterated analogs of NAEs and MGs was >99% for all compounds. Stock and working standard solutions were prepared in acetonitrile and stored at −20°C. While working standard solutions of up to 10 μg/ml were stable for protracted periods of time, a limited solubility was observed at a higher concentration (1 mg/ml) of stock solutions of saturated and monounsaturated NAEs and MGs after conservation at −20°C . Two mixtures of internal standards (ISTDs) were used: ISTD mix 1 prepared at 0.01 μg/ml AEA-d4, 0.01 μg/ml DHEA-d4, 0.02 μg/ml LEA-d4, 0.04 μg/ml PEA-d4, 0.04 μg/ml OEA-d4, 0.2 μg/ml 2-AG-d5, and 1 μg/ml 2-OG-d5; and ISTD mix 2 prepared at 5.0 μg/ml 2-AG-d8 and 0.25 μg/ml AEA-d8. The two 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**.

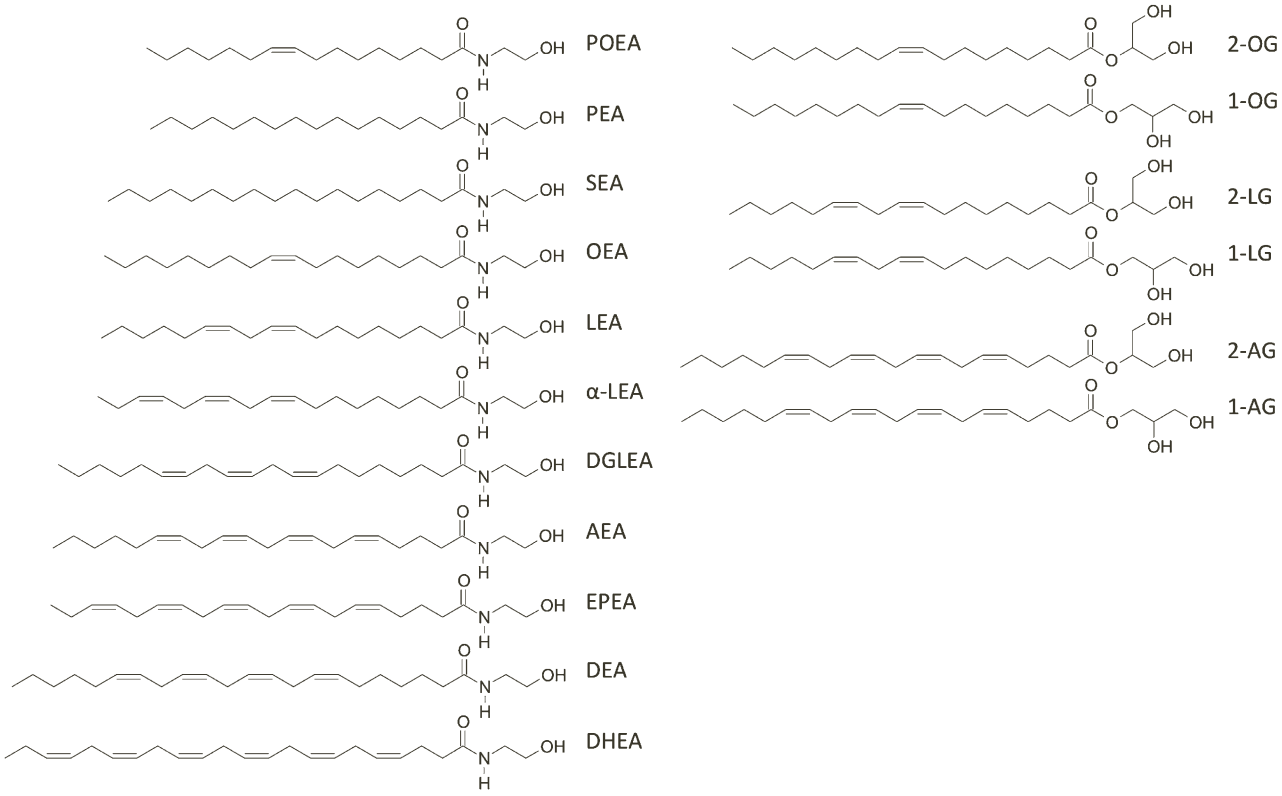

Fig. 1. Structures of the ECs and ERCs.

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/m^2 were recruited for the procurement of blood samples following the TANOBE 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.

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

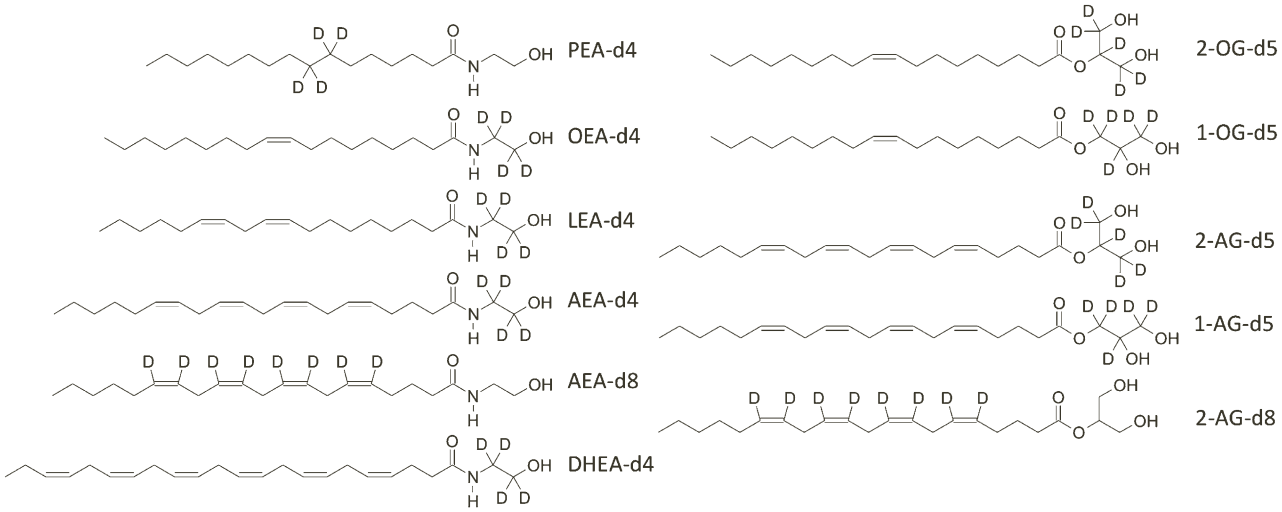

Fig. 2. Structures of the deuterated analogs of ECs and ERCs.

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[a]
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	323.5	7.26	0.07	324.5	62	44	135	12	LEA-d4	1.00
α-LEA	321.5	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.
[a]Response factor of the analyte versus the internal standard.

1 or mix 2, diluted up to 1 ml with 0.1 M Am. Ac. buffer (pH 4.0), extracted with 6 ml of TBME, and centrifuged (3,500 rpm, 5 min) at room temperature. The organic phase was transferred to clean tubes, evaporated (40°C, 20 min) under a stream of nitrogen, and extracts were reconstituted in 100 μl of a mixture of water:acetonitrile (10:90, v/v) with 0.1% formic acid (v/v) and transferred to HPLC vials. Twenty microliters were injected into the LC/MS-MS system.

LC/MS-MS analysis

An Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies, Wilmington, DE) equipped with a 1200 series binary pump, a column oven, and a cooled autosampler (4°C) was used. Chromatographic separation was carried out with a Waters C18-CSH column (3.1 × 100 mm, 1.8 μm particle size) maintained at 40°C with a mobile phase flow rate of 0.4 ml/min. The composition of mobile phase A was 0.1% (v/v) formic acid in water and mobile phase B was 0.1% (v/v) formic acid in acetonitrile. The initial conditions were 40% B. The gradient was first increased linearly to 90% B over 4 min, then increased linearly to 100% B over 5 min and maintained at 100% B for 3 min, to return to initial conditions for a further 4 min with a total run time of 16 min. The ion source was operated in the positive electrospray mode. A desolvation gas temperature of 350°C and a gas flow rate of 10 l/min were used. The pressure of the nebulizer was set at 40 psi and the capillary voltage at 4,000 V. The multiple reaction monitoring (MRM) mode was employed for quantification. The experimental MS conditions for each compound are listed in **Tables 1** and **2**.

Linearity

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: [EC]ng/ml = (ng ISTD × analyte response)/(ISTD response × RF × 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

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	66	135	12
AEA-d8	355.6	7.19	356.2	62	135	12
DHEA-d4	375.6	7.09	376.3	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	Ratio 2-AG-d5 (ISO1/ISO2)	Ratio 2-OG-d5 (ISO1/ISO2)
Spiked plasma		
TBME:water (6:1, v/v)[a]	0.59	0.32
TBME:water (6:1, v/v)[b]	0.53	0.20
TBME:Am. Ac. 0.1 M, pH 4.0 (6:1, v/v)[a]	0.10	0.09
Toluene:water (6:1, v/v)[a]	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.

[a] Reused clean tubes.

[b] New silanized tubes.

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

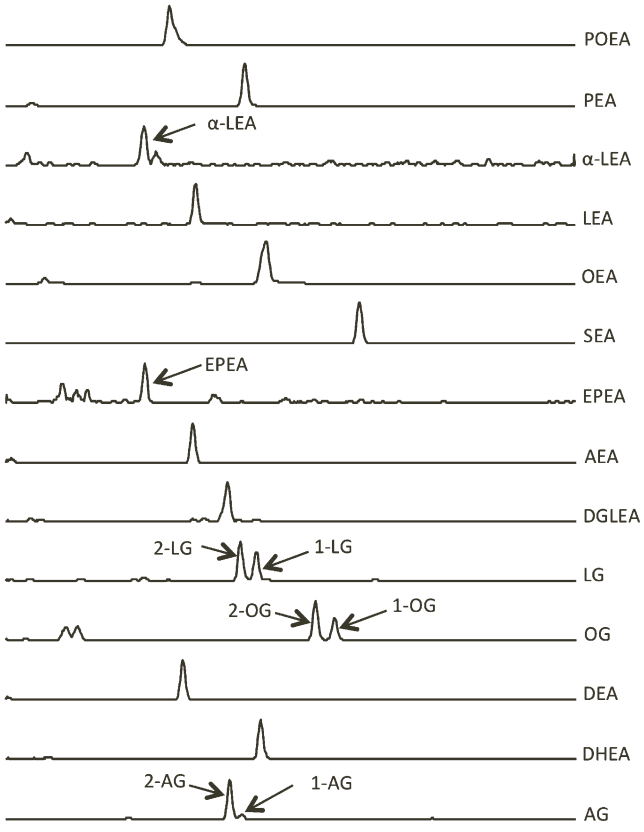

Fig. 3. LC/MS-MS chromatogram of EC and ERC profile of a human plasma sample.

isomer ratio was calculated. Additionally, in another experiment, the isomerization of endogenous MGs (2/1-AG, 2/1-LG, and 2/1-OG) was assessed in freshly obtained plasma samples from human volunteers preserved 2 h at 4ºC or room temperature.

Inhibition experiments of the ex vivo generation of MGs from plasma

Blood was obtained from human volunteers and processed immediately. The rate of production of MGs from the separated plasma was assessed by EC analysis after incubation for 2 h at RT under agitation. Basal EC levels (time 0) were estimated by immediate EC analysis. A set of potential inhibitors of MG production were tested by spiking the plasma before incubation for 2 h at RT at different concentrations of inhibitors. In several experiments, the following phospholipase and lipase inhibitors were tested: edelfosine, D609, FIPI, Orlistat, RHC 80267, KT172, 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 (IC_{50} = 9.6 μM). D609 is a phosphatidylcholine-specific phospholipase C inhibitor (K_i = 6.4 μM). FIPI is a phospholipase D_2 and D_1 inhibitor (IC_{50} = 20 and 25 nM, respectively). Orlistat is a gastric and pancreatic lipase inhibitor and a nonselective DAGLα and DAGLβ inhibitor (IC_{50} = 60 and 100 nM, respectively). RHC 80267 is a nonselective DAGL inhibitor (IC_{50} = 4 μM). KT172 and KT109 are selective DAGLβ inhibitors (IC_{50} = 60 and 42 nM, respectively) and also DAGLα inhibitors (IC_{50} = 0.14 and 2.3 μM, respectively). GSK 264220A is an endothelial lipase and a lipoprotein lipase inhibitor (IC_{50} = 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 IC_{50} 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 IC_{50}s for 2-AG, 2-LG, and 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 μg/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.

TABLE 5. Imprecision and accuracy

	Within Run						Between Run					
	Imprecision (%)			Accuracy (%)			Imprecision (%)			Accuracy (%)		
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
2-LG	11.0	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
2-OG	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
AEA	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	8.8	3.0	94.6	92.1	90.6	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	97.5	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

Data represent the mean values of QC sample replicates.

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 tubes) to test for the activity of catalytic silanols. Aqueous solvents were used to reduce viscosity and control plasma pH. Elevated temperatures, presence of serum albumin in the sample, and high pH values have been reported to accelerate 2-AG/1-AG acyl migration (13, 14, 17). It is worth noting that commercially available solutions of 2-AG and other 2-MG usually contain 5–10% 1-MG. Therefore, it seems impossible to completely avoid 2-MG/1-MG isomerization. The solvent that best preserved the original deuterated MG isomeric ratio of the sample after extraction was the mixture 6:1 TBME: 0.1 M Am. Ac. buffer (pH 4.0). In our experimental conditions, we observed that TBME preserved the 2-MG/1-MG ratio better than toluene, which was the solvent of choice for other authors (12, 14). However, this is most likely due to the longer evaporation time of toluene compared with TBME, which in our experiment was relevant due to the amount of solvent used (6 ml) in the liquid-liquid extraction. The standard solutions were prepared in acetonitrile because methanol and other protic solvents promote the isomerization of isomer 2 into isomer 1 of MG (13). No differences were observed between extraction tubes that were 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

TABLE 6. Stability of MG concentrations on different collection conditions

		2/1-AG		2/1-LG		2/1-OG	
Blood Tube	Inhibitor in Plasma	Time 0	Time, 2 h at room temperature	Time 0	Time, 2 h at room temperature	Time 0	Time, 2 h at room temperature
EDTA	−	0.75 ± 0.21	8.51 ± 4.57	8.57 ± 2.23	102 ± 86.5	9.68 ± 2.28	115 ± 85.9
EDTA	+		1.13 ± 1.03		11.8 ± 5.1		15.6 ± 2.30
Heparin	−	2.82 ± 0.67	30.6 ± 23.0	83.7 ± 12.8	876 ± 729	72.3 ± 0.93	619 ± 475
Heparin	+		2.99 ± 0.05		65.3 ± 3.77		48.0 ± 1.20

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.

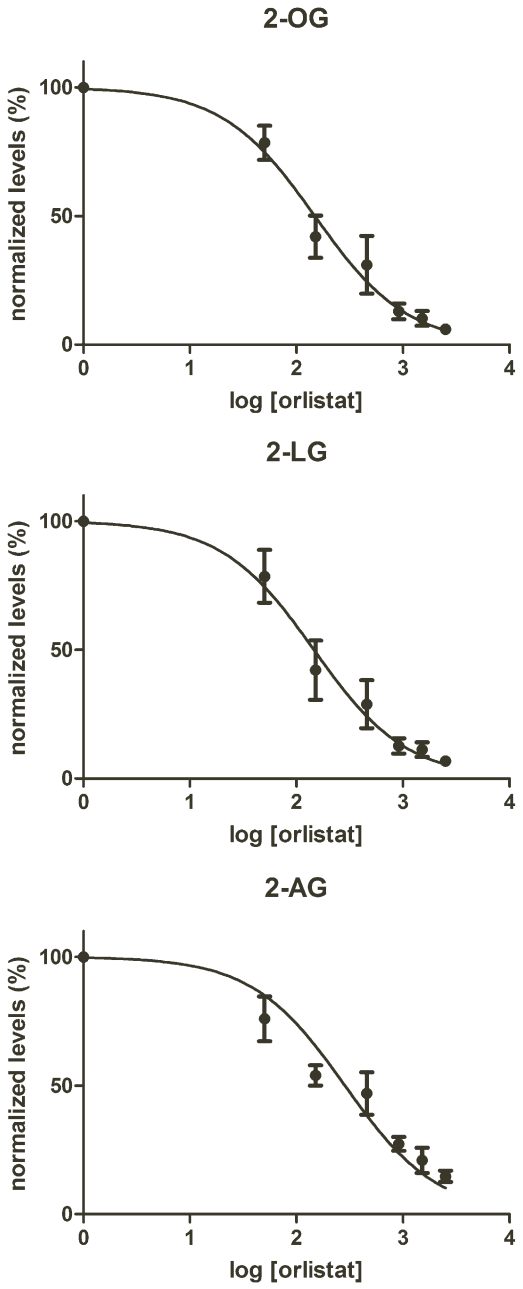

Fig. 4. Inhibition model of ex vivo production of 2-MG in plasma with Orlistat. Plasma from freshly extracted blood was spiked with a range of concentrations of Orlistat, incubated at room temperature for 2 h, and analyzed by LC/MS-MS to detect the levels of 2-AG, 2-OG, and 2-LG. Values represent the normalized 2-MG levels of three different volunteers analyzed in triplicate.

to glycerol, is common to the fragmentation of MGs. The specific MRM transitions are listed in Tables 1 and 2. The method is selective and specific for each analyte with no cross contamination between MRM channels and, in most cases, a single chromatographic peak corresponding to the endogenous analyte was found throughout the acquisition time. The variation in retention time of the individual analytes in a typical batch analysis was less than 0.5%. The retention time and product ion spectra of the endogenous analytes in the matrix matched the ones of the authentic standards.

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

TABLE 7. Effect of Orlistat addition to plasma on EC measures

EC/ERC	n	Measured Concentration (ng/ml)		Change (%)	*P*
		Without Added Orlistat	With Added Orlistat		
2/1-AG	25	2.42 ± 1.10	0.89 ± 0.50	−59.0 ± 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	−2.09 ± 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	0.03 ± 0.01	0.03 ± 0.01	−8.67 ± 9.02	0.104
LEA	25	1.30 ± 0.38	1.23 ± 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.

approximately 5-fold increases in 2/1-LG and 2/1-OG concentrations in plasma preserved for just 2 h at room temperature compared with plasma preserved at 4°C. In regard to 2/1-AG, we observed differences in concentration when plasma was analyzed immediately after blood extraction versus some time afterwards (2 h), or after a freezing/thawing step even though cold chain (4°C) was maintained. We therefore investigated, in controlled experiments, several phospholipase and lipase inhibitors for their capacity to inhibit ex vivo MG production in plasma. We found no inhibition activity for the phospholipase inhibitors D609, FIPI, and edelfosine (IC_{50} >30 μM, IC_{50} >125 nM, and IC_{50} >50 μM, respectively). On the other hand, we observed inhibition of the ex vivo generation of 2-AG, 2-LG, and 2-OG in plasma spiked with Orlistat, a gastric and pancreatic lipase inhibitor (22) and a potent nonspecific inhibitor of DAGLα and DAGLβ (23). It is to be noted that the artifactual generation of MGs persisted even after immediate sample centrifugation that essentially eliminates all blood cells, which means that this MG buildup should be related to an enzymatic plasma activity. Because Orlistat is a general lipase inhibitor, we also tested the inhibition activity of RHC 80267, another nonspecific inhibitor of DAGLα (23), KT172, and KT109, potent selective DAGLβ inhibitors (24). None of these compounds showed inhibition activity (IC_{50} >30 μM, IC_{50} >15 μM, and IC_{50} >15 μM, respectively), which means that the ex vivo MG production in plasma is a mechanism independent 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 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

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

Spiked analyte		Ratio			
		Time 0	Time, 30 min at room temperature	Time, 20 days at −20°C	Time, 20 days at −80°C
2-AG-d5	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-d5	ISO1/ISO2	0.08 ± 0.01	0.36 ± 0.06	0.58 ± 0.03	0.36 ± 0.06
1-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.

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

	Ratio ISO1 / ISO2	
Endogenous analyte	Time 2 h at 4°C	Time 2 h at room temperature
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),

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 biomarker, the understanding of this apparent alternative biosynthetic pathway of 2-AG, probably linked to lipid metabolism, would contribute to a better comprehension of the significance of its blood concentrations.

We have validated a method for the determination of a range of MGs and NAEs in plasma. The developed method is able to preserve the original isomeric ratio of MGs. We have found that the chemical isomerization of MGs can only be avoided by immediate processing (at cold temperature and acid pH) and analysis of samples. The report of MGs as the sum of both isomers may be considered. Alternatively, appropriate isomerization controls can be used during sample collection and conservation in order to correct concentrations. Data suggest that isomer 1 of AG is not an endogenous compound, and most probably is the result of chemical isomerization during storage and sample processing. On the other hand, isomer 1 of OG and isomer 1 of LG are likely to be endogenous compounds that result from in vivo isomerization that takes places during digestion, and their concentrations can be detected in plasma.

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