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Gas chromatography–mass spectrometry of *cis*-9,10-epoxyoctadecanoic acid (*cis*-EODA) II. Quantitative determination of *cis*-EODA in human plasma

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Abstract

Cytochrome P450 dependent epoxidation and non-enzymic lipid peroxidation of oleic acid (*cis*-9-octadecenoic acid) result in the formation of *cis*-9,10-epoxyoctadecanoic acid (*cis*-EODA). This oleic acid oxide has been identified indirectly in blood and urine of humans. Reliable concentrations of circulating *cis*-EODA have not been reported thus far. In the present article, we report on the first GC–tandem MS method for the accurate quantitative determination in human plasma of authentic *cis*-EODA as its pentafluorobenzyl (PFB) ester. *cis*-[9,10-$^2H_2$]-EODA (*cis*-d_2-EODA) was synthesized by chemical epoxidation of commercially available *cis*-[9,10-$^2H_2$]-9-octadecenoic acid and used as an internal standard for quantification. Endogenous *cis*-EODA and externally added *cis*-[9,10-$^2H_2$]-EODA were isolated from acidified plasma samples (1 ml; pH 4.5) by solvent or solid-phase extraction, converted into their PFB esters, isolated by HPLC and quantified by selected reaction monitoring. The parent ions $[M–PFB]^-$ at mass-to-charge ratio (*m*/*z*) 297 for *cis*-EODA and *m*/*z* 299 for (*cis*-d_2-EODA) were subjected to collisionally-activated dissociation and the corresponding characteristic product ions at *m*/*z* 171 and 172 were monitored. In plasma of nine healthy humans (5 females, 4 males), *cis*-EODA was found to be present at 47.6 ± 7.4 nM (mean ± S.D.). Plasma *cis*-EODA levels were statistically insignificantly different ($P = 0.10403$, *t*-test) in females (51.1 ± 3.4 nM) and males (43.1 ± 2.2 nM). *cis*-EODA was identified as a considerable contamination in laboratory plastic ware and found to contribute to endogenous *cis*-EODA by approximately 2 nM. The present GC–andem MS method should be useful in investigating the physiological role(s) of *cis*-EODA in humans.

Keywords: *cis*-9,10-Epoxyoctadecanoic acid; Oleic acid

1. Introduction

The major monounsaturated fatty acid in mammals is oleic acid, i.e. *cis*-9-octadecenoic acid (Fig. 1). To date only little attention has been paid to the physiology, pathology and metabolism of oleic acid. However, several studies suggest potential physiological and pathological roles for oleic acid [1–12]. It is still unknown whether the oleic acid-induced effects are attributed to oleic acid itself or to biologically active oleic acid metabolites.

Ulsaker and Teien have shown that the oleic acid oxide, i.e. *cis*-9,10-epoxyoctadecanoic acid (*cis*-EODA; Fig. 1), is physiologically present in human blood [10,11]. More recently, the same group has identified *cis*-EODA in human urine and estimated its concentration to be 2.1 nM by GC–MS [12]. This concentration is of the same order of magnitude of that of epoxyeicosatrienoic acids, i.e. oxidative metabolites of arachidonic acid, in human urine [13]. The identification of *cis*-EODA in vivo elucidates the metabolic pathway of oleic acid [10–12].

The origin of circulating and excretory *cis*-EODA is unknown. Ulsaker and Teien have assumed that urinary EODA is a real metabolic product of oleic acid excreted by the kidney, and not an artefact formed from urinary oleic acid during sample treatment [12]. *cis*-EODA can be produced from oleic acid by enzymic and non-enzymic epoxidation. Oleic acid, both unesterified and esterified to lipids, undergoes non-enzymic oxidative metabolism, which leads to

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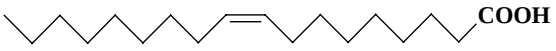

***cis*-9-octadecenoic acid (oleic acid)**

***cis*-9,10-epoxyoctadecanoic acid (*cis*-EODA)**

Fig. 1. Chemical structures of *cis*-9-octadecenoic acid (oleic acid) and its oxide, i.e. *cis*-9,10-epoxyoctadecanoic acid (*cis*-EODA).

formation of the free and the esterified forms of *cis*-EODA [14–17]. The detection of racemic *cis*-EODA in lipids of human leukocytes [17] suggests a free radical-catalyzed epoxidation of esterified oleic acid in lipids. On the other hand, the cytochrome P450 (CYP) system has been shown to be responsible for the enzymic epoxidation of oleic acid to *cis*-EODA [18,19]. The CYP isoforms 2C2 and 2CAA have been shown to epoxidize oleic acid to *cis*-EODA [20].

The presence of *cis*-EODA in human blood and urine has been shown only indirectly, i.e., after reduction of the epoxy and carboxy groups with $LiAlH_4$ to generate 1,9-dihydroxyoctadecane and 1,10-dihydroxyoctadecane [10–12]. However, *cis*-EODA is stable enough in order to be isolated from biological fluids prior to GC–MS analysis [10–12]. Furthermore, in previous work we showed that the oxirane ring of *cis*-EODA is thermally sufficiently stable for GC–MS analysis of intact *cis*-EODA as its methyl or pentafluorobenzyl (PFB) ester [19]. The possibility of analyzing intact *cis*-EODA as its PFB ester by GC–MS in the negative-ion chemical ionization (NICI) mode fulfills an important requirement for sensitive quantitative determination of *cis*-EODA in biological fluids, such as plasma, serum and urine of humans, in which basal levels of *cis*-EODA are expected to be in the lower nM-range [10–12].

In the present study, we report on a fully-validated GC–tandem MS method for the quantitative determination of *cis*-EODA in human plasma as the PFB ester of the intact molecule. For this purpose, we synthesized and used *cis*-[9,10-$^2H_2$]-EODA (*cis*-d_2-EODA) as internal standard. We report here on the first accurately measured basal levels of *cis*-EODA in plasma of humans.

2. Experimental

2.1. Chemicals

Oleic acid, *cis*-9,10-epoxyoctadecanoic acid (*cis*-EODA), *trans*-9,10-epoxyoctadecanoic acid (*trans*-EODA), *N*,*N*-diisopropylethylamine (99.5%), potassium superoxide (KO_2), 30 and 85 vol.% H_2O_2, dichloromethane and acetic anhydride were obtained from Sigma (Taufkirchen, Germany). These chemicals were of the highest purity available (≥98%). *cis*-[9,10-$^2H_2$]-9-Octadecenoic acid (98 at.% ^2H) was supplied by Isotec Inc. (Miamisburg, Ohio, USA). Acetonitrile and methanol of gradient grade, acetonitrile and methanol of *SupraSolv* quality, and ethanol of *SeccoSolv* quality were obtained from Merck (Darmstadt, Germany). Water of HPLC quality was purchased from Baker (Deventer, The Netherlands). 2,3,4,5,6-Pentafluorobenzyl bromide (PFB-Br, 99%) and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, 99%) were obtained from Pierce (Rockford, Ill, USA). For solid-phase extraction (SPE), two different SPE columns filled with end-capped octadecyl-modified silica (C18ec) from Macherey-Nagel (Düren, Germany) were used. SPE columns were made either of glass (Chromabond C18ec; 6 ml, 500 mg) or of polypropylene (Chromabond-LV C18ec; 15 ml, 500 mg). Immunoaffinity chromatography (IAC) columns (8-isoprostane affinity column; 4 ml containing 1 ml gel resin) with immobilized antibodies against 8-*iso*-$PGF_{2\alpha}$ were obtained from Cayman Chemicals (Ann Arbor, MI, USA).

cis-d_2-EODA was obtained from *cis*-[9,10-$^2H_2$]-9-octadecenoic acid epoxidation by means of the synthetic use of peracetic acid [21]. Peracetic acid was prepared at room temperature from acetic anhydride (1 ml) and H_2O_2 (305 mg of 85 vol.% H_2O_2) using dimethylaminopyridine (0.25 mg) as a catalyst. *cis*-[9,10-$^2H_2$]-9-octadecenoic acid (65.6 mg, 0.231 mmol) was dissolved in dichloromethane (2 ml), peracetic acid (0.3 ml) was added in two portions at 0–5 °C within 1 h, and the reaction was monitored by silica TLC using *tert*-butyl methyl ether-hexane (1:2, v/v) as the mobile phase. After 3 h of incubation at room temperature the epoxidation was complete. All volatiles were removed in high vacuum. *cis*-[9,10-$^2H_2$]-EODA was obtained in quantitative yield as a white solid with a melting point of 56 °C. IR analysis (FT–IR-Vector 22 from Bruker) of the preparation gave ν = 3200–2400 (broad), 2916, 2849, 2209 (C–^{2}H bond), 1696, 1443, 1304, 1266, 1226, 1193 and 913 cm^{-1}. ^1H NMR analysis (AM 400 from Bruker, $CDCl_3$/TMS) yielded signals with a chemical shift (δ (ppm)) at 3.0, s, broad, OH; 2.35, t (J = 7.5 Hz) CH_2; 1.64, quint (J = 7.0 Hz) CH_2; 1.6–1.2, 3 m, 12 CH_2 groups, and 0.88, t (J = 7.0 Hz) CH_3. In the ^1H NMR spectrum of *cis*-[9,10-$^2H_2$]EODA no signal was obtained at 2.90 ppm for unlabelled *cis*-EODA.

2.2. Analytical procedures

Venous blood anticoagulated with EDTA or citrate or lithium heparin was obtained from healthy non-smoking volunteers. Plasma was prepared immediately by centrifugation (800 × *g*, 5 min, 4 °C), proportioned in 1 ml aliquots and stored at −80 °C until analysis. Urine was obtained from spontaneous micturition, proportioned in 20 ml aliquots and stored at −20 °C until analysis. To minimize sample contamination by exogenous *cis*-EODA present in laboratory materials, synthetics was replaced by glass ware where possible, including SPE columns, pipettes and syringes, and organic solvents of *SupraSolv* or *SeccoSolv* quality as well as water

of HPLC grade were used. The extraction procedures outlined below for plasma samples were also applied to urine samples, for which the internal standard was added at a final concentration of 5 nM. Other divergences from the procedure described below are discussed in Section 3.

2.2.1. Solvent extraction procedure

cis-d_2-EODA (10 pmol, i.e. 20 μl of a 5 μM solution in methanol; or 50 pmol, i.e. 50 μl of a 1 μM solution in methanol) is added to plasma (1 ml), the sample is mixed by vortexing and let to equilibrate for 30 min in an ice-bath. The sample is acidified to pH 4–5 by 20 vol.% acetic acid. Use of stronger acids such as hydrochloric acid should not be used in order to avoid lower pH values at which the epoxide is unstable. Ethyl acetate (2 ml) is added, and the mixture is mixed by vortexing for 2 min. After centrifugation (800 × *g*, 2 min, 4 °C), the upper layer is decanted, dried over anhydrous sodium sulfate, transferred into glass vials, and the solvent is evaporated to dryness under a stream of nitrogen. Derivatization is performed as described below.

2.2.2. Solid-phase extraction procedure

cis-d_2-EODA (10 pmol, i.e. 20 μl of a 5 μM solution in methanol; or 50 pmol, i.e. 50 μl of a 1 μM solution in methanol) is added to plasma (1 ml), the sample diluted with distilled water (4 ml), mixed by vortexing and let to equilibrate for 30 min in an ice-bath. The sample is acidified to pH 4–5 by 20 vol.% acetic acid and applied to a SPE column preconditioned with methanol (10 ml) and distilled water (5 ml). SPE column is washed with distilled water (10 ml) and air-dried. Compounds are eluted with ethyl acetate (5 ml). Sample volume is reduced to approximately 1 ml under a stream of nitrogen, and the remaining organic phase is transferred to a glass vial. Solvents are evaporated to dryness and derivatization is performed as described below.

2.2.3. Derivatization procedures

PFB esters of fatty acids were prepared by a standard derivatization procedure [19]. Briefly, solvents are removed by a stream of nitrogen gas and residues are reconstituted in water-free acetonitrile (100 μl). *N*,*N*-Diisopropylethylamine (20 μl) and PFB-Br (20 μl from a solution of PFB-Br in acetonitrile; 30% (v/v)) are added, and the samples are incubated at 30 °C for 60 min.

Prior to GC–MS analysis, solvents and reagent excess are removed under nitrogen, residues are reconstituted in BSTFA (50 μl), and samples are heated at 60 °C for 15 min. This derivatization step is not absolutely required for the GC–MS analysis of *cis*-EODA. However, silylation of hydroxylated co-extracted compounds improves GC analysis of *cis*-EODA (data not shown). Samples are stored in BSTFA at 4 °C until analysis.

2.2.4. HPLC analysis of PFB esters

Separation of PFB esters by HPLC was carried out on LKB apparatus consisting of a pump Model 2150, a controller Model 2152, a solvent conditioner Model 2156, a Rheodyne injection system fitted with a 200 μl sample loop, and of a variable UV-Vis LKB detector Model 2151. Chromatogram recordings and calculations were performed on Shimadzu integrator Model C-R3A. The analytical columns used (250 mm × 4.6 mm i.d.) were packed with Nucleosil 100-5C_{18}, 5 μm particle size, from Macherey-Nagel (Düren, Germany). The mobile phase consisted of acetonitrile–water (70:30 (v/v)) and was pumped at a flow rate of 2 ml/min. For GC–MS analysis, the HPLC fraction corresponding to the PFB ester of *cis*-EODA is collected (typically 11.5 min), distilled water is added (1 ml), and PFB esters are extracted by vortexing for 1 min with ethyl acetate (2 ml). The organic phase is decanted and dried over anhydrous sodium sulfate, the solvent removed under nitrogen, and the residue treated with BSTFA (50 μl) as described above.

2.2.5. GC–MS and GC–tandem MS analyses

GC–MS and GC–tandem MS analyses were performed on a Thermoquest TSQ 7000 triple-stage quadrupole mass spectrometer interfaced with a Thermoquest gas chromatograph model Trace 2000 which was equipped with programmed temperature evaporation (PTV) injector and an autosampler model AS 2000 (Egelsbach, Germany). Fused silica capillary columns Optima 17 (30 m × 0.25 mm i.d., 0.25 μm film thickness) from Macherey-Nagel (Düren, Germany) were used. Helium was used as a carrier gas at a constant flow of 1 ml/min. For NICI methane was used as a reagent gas at a pressure of 65 Pa. Argon was used for collisionally-activated dissociation (CAD) at a pressure of 0.15 Pa. The collision energy was set to 25 eV. Electron energy was 200 eV and the emission current 600 μA. Interface and ion source were kept at 290 and 180 °C, respectively. In quantitative analyses, sample injection (1 μl, splitless) was performed by PTV starting at an injector temperature of 80 °C which was increased to 280 °C at 10°/s. The column was held at 80 °C for 2 min, then programmed to 340 °C at 8°/min. Quantification by GC–MS was performed by selected ion monitoring (SIM) of the parent ions $[M–PFB]^-$ at mass-to-charge ratio (*m*/*z*) 297 for *cis*-EODA and *m*/*z* 299 for *cis*-d_2-EODA with a dwell time of 400 ms for each ion. In GC–tandem MS, the product ions at *m*/*z* 171 for *cis*-EODA and *m*/*z* 172 for *cis*-d_2-EODA generated by CAD of the parent ions $[M–PFB]^-$ at *m*/*z* 297 and 299, respectively, were monitored in the selected reaction monitoring (SRM) mode with a dwell time of 400 ms for each ion.

3. Results

3.1. GC–MS and GC–tandem MS mass spectra of *cis*-EODA and *cis*-d_2-EODA

The NICI mass spectra of the PFB esters of *cis*-EODA (Fig. 2A) and *cis*-d_2-EODA (Fig. 2C) show few intense mass fragments. The most intense ions were observed at

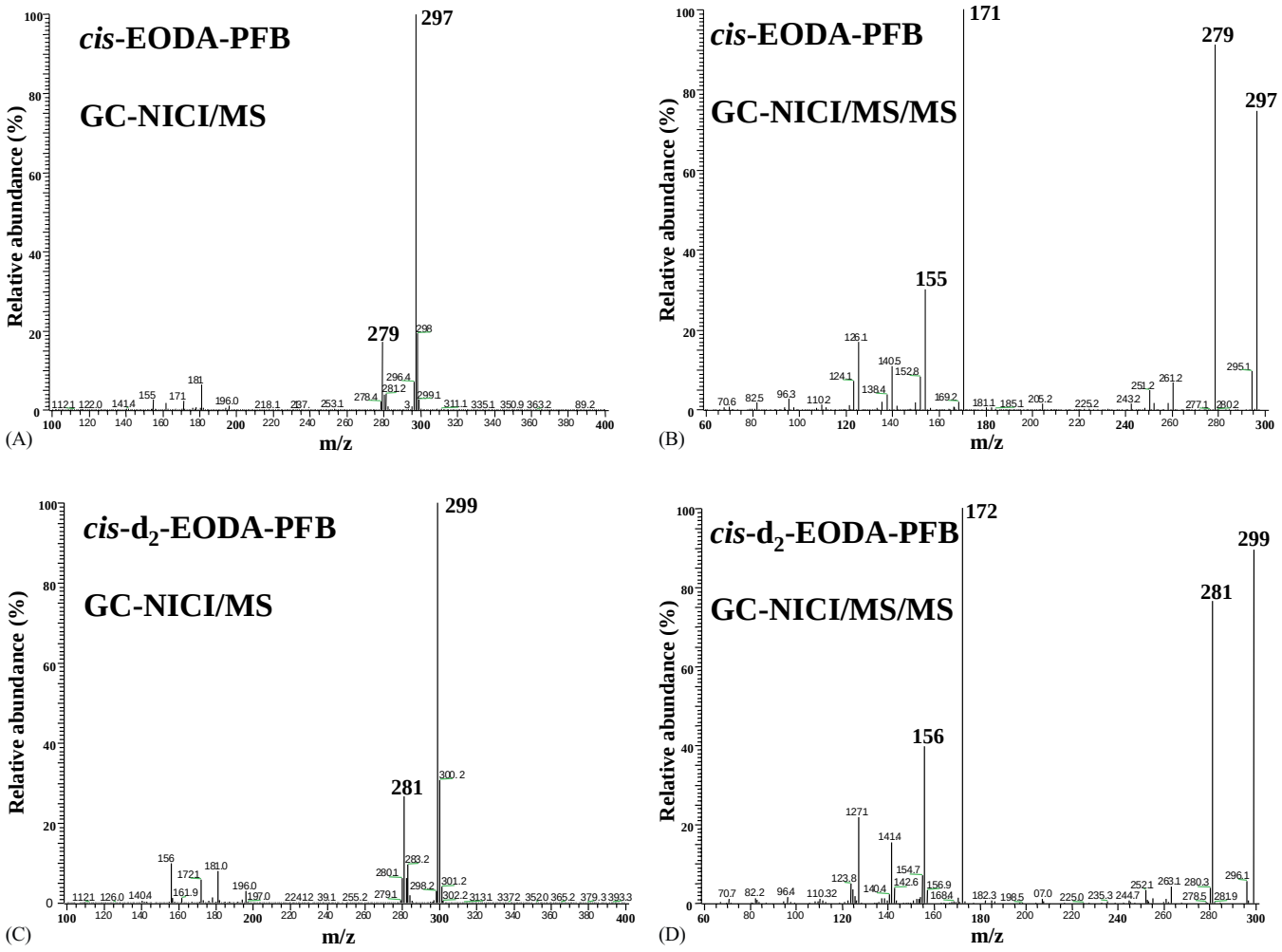

Fig. 2. Negative-ion chemical ionization (NICI) mass spectra (MS) and tandem mass spectra (MS/MS) of the pentafluorobenzyl (PFB) esters of authentic *cis*-9,10-epoxyoctadecanoic acid (*cis*-EODA) and *cis*-9,10-epoxy-[9,10-$^2H_2$]octadecanoic acid (*cis*-d_2-EODA). Tandem mass spectra were obtained by collision-activated dissociation of the parent ions $[M\text{-}PFB]^-$ at *m*/*z* 297 for *cis*-EODA and *m*/*z* 299 for *cis*-d_2-EODA at a collision energy of 25 eV.

297 for *cis*-EODA and *m*/*z* 299 for *cis*-d_2-EODA, and result from loss of PFB radicals from the carboxylic PFB esters, i.e. they correspond to the parent carboxylate anions $[M–PFB]^-$. Less intense mass fragments were observed at *m*/*z* 279 for *cis*-EODA and *m*/*z* 281 for *cis*-d_2-EODA. These anions result from the corresponding parent ions by neutral loss of each 18 Da, most likely due to loss of the oxirane O atom and loss of two H atoms from C atoms other than C9 and C10.

CAD of the parent ions $[M–PFB]^-$ at *m*/*z* 297 and 299 resulted also in the formation of the ions at *m*/*z* 279 and 281 (Fig. 2B and D). The most intense and most characteristic product ions were observed at *m*/*z* 171 and 155 for *cis*-EODA, and at *m*/*z* 172 and 156 for *cis*-d_2-EODA. The latter are each 1 Da larger than those of *cis*-EODA, indicative of the presence of each 1 deuterium atom in the ions with *m*/*z* 172 and 156. The difference of 16 Da between *m*/*z* 171 and 155 as well as *m*/*z* 172 and 156 reveals that the product ions at *m*/*z* 171 and 172 carry the epoxide O atoms in their molecules. These observations strongly suggest that the ions at *m*/*z* 171 and 172, and the ions at *m*/*z* 155 and 156 are produced by cleavage of the carboxylate anions between C9 and C10 of their parent ion molecules. From the PFB ester of *trans*-EODA there were generated parent mass spectra and product ion mass spectra very similar to those of *cis*-EODA (data not shown). Therefore, the product ions at *m*/*z* 171 and 155 are characteristic for 9,10-epoxyoctadecanoic acid. Discrimination between the PFB esters of *cis*- and *trans*-EODA is possible both by GC and HPLC [19]. In the present study the relative GC retention time of *cis*- to *trans*-EODA was 1.04 when analyzed as PFB ester (see also below).

The base peak in the EI mass spectrum of the PFB ester of *cis*-EODA (not shown) was observed at *m*/*z* 181 (100%) corresponding to the PFB cation. Other mass fragments were found at *m*/*z* 365 (40%) (cleavage between C10 and C11) and *m*/*z* 155 (40%) (cleavage between C8 and C9). The corresponding ions in the EI mass spectrum the PFB ester of *cis*-d_2-EODA (not shown) were *m*/*z*: 181 (100%), 367 (40%) and 156 (40%). These ions strongly suggest that the PFB esters of *cis*-EODA and *cis*-d_2-EODA carry the intact

oxirane groups at C9–C10. The molecular cations of the PFB esters of *cis*-EODA and *cis*-d_2-EODA were also observed (*m*/*z* 487 and 489, respectively, but they were of very weak intensity (<1%). CAD of the parent ions at *m*/*z* 365 and 367 generated each a single product ion at *m*/*z* 181, i.e. the PFB cation (not shown). Presumably, GC–tandem MS analysis of *cis*-EODA as PFB ester in the EI mode, unlike in the NICI mode, would not further enhance the method's selectivity.

3.2. Standardization of cis-d_2-EODA

Newly synthesized *cis*-d_2-EODA (100 pmol) was converted to its PFB ester and analyzed by GC–tandem MS in the SRM mode. The chromatogram shown in Fig. 3 indicates that the *cis*-d_2-EODA preparation contains only very small amounts of unlabelled *cis*-EODA (see also Fig. 2C). The peak area ratio of *m*/*z* 171 to 172 from *cis*-d_2-EODA was determined by triplicate analysis to be 0.00302 with a precision (R.S.D.) of 5.6%, indicating presence of *cis*-EODA in the *cis*-d_2-EODA preparation at a portion of approximately 0.3%. The peak area ratio of *m*/*z* 172 to 171 from unlabelled *cis*-EODA was determined by triplicate analysis to be 0.0176 with a precision (R.S.D.) of 2.3%. The ratio of 0.0176 indicates a contribution of approximately 1.8% of *cis*-EODA to *cis*-d_2-EODA under these GC–tandem MS conditions.

Stock solutions of commercially available *cis*-EODA and newly synthesized *cis*-d_2-EODA were prepared in methanol and stored at −20 °C. Mixtures containing varying amounts of *cis*-d_2-EODA (0–200 pmol) and a fixed amount of *cis*-EODA (75 pmol) were derivatized and analyzed by SRM

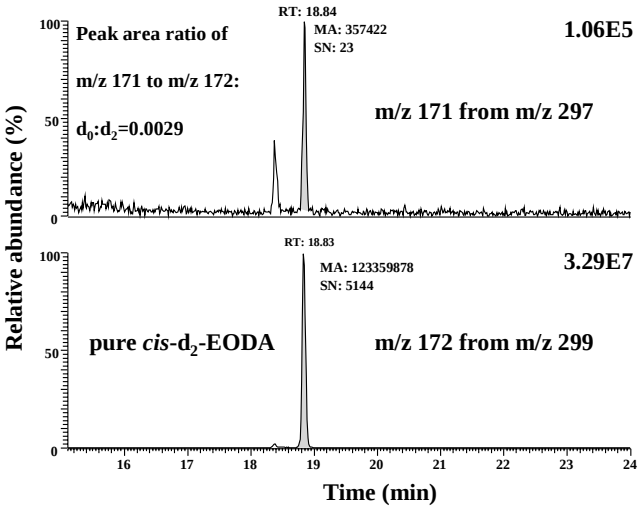

Fig. 3. A partial chromatogram from the GC–tandem MS analysis of newly synthesized *cis*-d_2-EODA (100 pmol) as pentafluorobenzyl ester which emerged from the column at 18.83 min (underlaid peak). Selected reaction monitoring of *m*/*z* 171 from *m*/*z* 297 for unlabelled *cis*-EODA (upper panel) present in the preparation and of *m*/*z* 172 from *m*/*z* 299 for *cis*-d_2-EODA (lower panel). The ratio of areas of the peaks eluting at 18.84 and 18.83 min with *m*/*z* 171 and 172, respectively, was determined in this sample to be 0.0029. Sample was injected at an injector temperature of 280 °C.

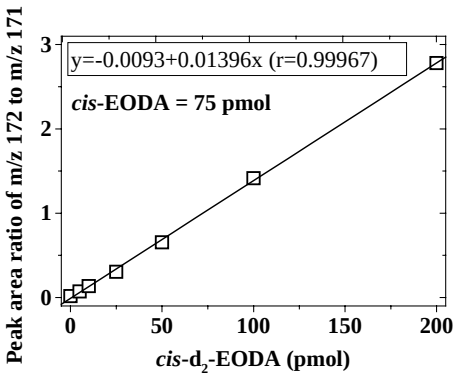

Fig. 4. Standardization of newly synthesized and weighed *cis*-d_2-EODA by GC–tandem MS by using weighed unlabelled *cis*-EODA. Mixtures containing a fixed amount of *cis*-EODA (75 pmol) and varying amounts of *cis*-d_2-EODA (0, 5, 10, 25, 50, 100, and 200 pmol) were derivatized and analyzed by selected-reaction monitoring of *m*/*z* 172 from *m*/*z* 299 for *cis*-d_2-EODA and of *m*/*z* 171 from *m*/*z* 297 for *cis*-EODA. The peak area ratio of *m*/*z* 172 to 171 was calculated and plotted versus the amount of *cis*-d_2-EODA. Samples were injected at an injector temperature of 280 °C.

of *m*/*z* 171 and 172. Linear regression analysis between the peak area ratio of *m*/*z* 172 to 171 (y) and the varying amount of *cis*-d_2-EODA (x) resulted in a straight line with the regression equation $y = -0.0093 + 0.01396x$, $r = 0.999$ (Fig. 4). This finding indicates the utility of *cis*-d_2-EODA as an internal standard for *cis*-EODA in GC–tandem MS. The reciprocal of the slope of the regression equation, which corresponds to the amount of *cis*-EODA used in this experiment, amounts to 71.6 pmol which agrees by 95.5% with the amount of 75 pmol from weighed unlabelled *cis*-EODA.

3.3. Validation of the method

3.3.1. Chemical interferences

Basal levels of *cis*-EODA were determined at first by GC–tandem MS both in the NICI and EI mode by using *cis*-d_2-EODA at a final concentration of 10 nM for plasma and urine samples and by means of the solvent extraction procedure. Triplicate analysis of a plasma sample (1 ml) yielded a *cis*-EODA concentration of 54.8 nM with a precision (R.S.D.) of 21.9% in the EI mode (SRM of *m*/*z* 181 from *m*/*z* 365 to 367) and 58.6 nM with a precision of 3.5% in the NICI mode (SRM of *m*/*z* 171 from *m*/*z* 297, and of *m*/*z* 172 from *m*/*z* 299). In the EI mode many peaks were obtained in addition to that of *cis*-EODA, the intensity of which was several times smaller than that in the NICI mode (not shown).

Triplicate analysis of another plasma sample (1 ml) in the NICI mode yielded a *cis*-EODA concentration of 63.8 nM with a precision (R.S.D.) of 4.0% by SRM of *m*/*z* 171 from *m*/*z* 297 and *m*/*z* 172 from *m*/*z* 299. By SRM of *m*/*z* 155 from *m*/*z* 297 and of *m*/*z* 156 from *m*/*z* 299, the concentration of *cis*-EODA in this plasma sample was determined to be 63.2 nM with a precision of 3.6%. Thus, quantification of *cis*-EODA in plasma by SRM of both product ions occur

with almost the same precision and presumably with the same accuracy. However, SRM of the products ions at *m*/*z* 171 and 172 provided only two peaks corresponding to PFB esters of *cis*- and *trans*-EODA, while SRM of *m*/*z* 151 and 152 resulted in additional peaks. Furthermore, the signal-to-noise (S/N) ratio was approximately eight times higher when detecting *m*/*z* 172 as compared with *m*/*z* 156. SRM of the product ions at *m*/*z* 279 and 281 did not allow accurate and precise quantification of *cis*-EODA (data not shown). For these reasons, the method's validation and quantitative analyses were performed in the NICI by SRM of *m*/*z* 171 from *m*/*z* 297 for *cis*-EODA and of *m*/*z* 172 from *m*/*z* 299 for the internal standard *cis*-d_2-EODA. The LOD of the method in the SRM mode under NICI conditions was approximately 20 fmol of *cis*-EODA (S/N ratio of 6:1).

Oxidation of plasma oleic acid to EODA during the sample treatment was simulated by using two strong oxidizing chemicals, i.e. H_2O_2 and superoxide which was supplied as pure KO_2. Pooled freshly obtained human plasma was spiked with *cis*-d_2-EODA at a final concentration of 100 nM and proportioned in 1 ml aliquots. One sample was treated without any addition of H_2O_2 and, four samples were spiked with H_2O_2 to achieve final concentrations of 10, 50, 100 and 1000 μM. Quantification by GC–tandem MS yielded *cis*-EODA concentrations of 40.8 nM in the unspiked plasma sample, and 41.2, 40.1, 38.4 and 40.1 nM in the spiked plasma samples, indicating no H_2O_2-dependent formation of *cis*-EODA from plasma oleic acid during the extraction and derivatization procedures. Addition of KO_2 (approximately 5 mg) to three 1 ml aliquots of fresh plasma with a basal *cis*-EODA concentration of 60 nM did not result in additional formation of *cis*-EODA (59.2, 59.4 and 61.7 nM in spiked plasma) as measured by GC–tandem MS. However, addition of *cis*-[9,10-$^2H_2$]-9-octadecenoic acid to plasma at a final concentration of 100 μM followed by addition of approximately 5 mg of KO_2 yielded formation of 5.9 nM *cis*-d_2-EODA. As *cis*-EODA may be formed artifactually from oxidation of plasma oleic acid only under very drastic conditions, no special precautions were taken to avoid oleic acid autoxidation during the whole method.

3.3.2. Accuracy and precision

A pooled plasma sample containing *cis*-EODA at basal concentration of 58.2 nM was used to determine accuracy (recovery) and precision of the method in a relevant concentration range, e.g. up to 240 nM added concentration of synthetic *cis*-EODA. Plasma samples (each three 1 ml aliquots) were spiked with *cis*-d_2-EODA at a fixed final concentration of 10 nM and varying concentrations of *cis*-EODA. The results of this experiment are summarized in Table 1 and indicate the highly accuracy and precision of the method for *cis*-EODA in human plasma. Linear regression analysis between measured (*y*) and added (*x*) *cis*-EODA concentrations resulted in the regression equation $y = 60.6 + 0.924x$ and $r = 0.999$.

Table 1
Data on the validation of the method[a] for *cis*-EODA in human plasma[b]

cis-EODA added (nM)	Measured		Recovery (%)	Precision (R.S.D., %)
	Mean	S.D.		
0.00	58.6	0.2	N.A.	3.5
10.0	67.5	2.2	88.7	3.2
30.0	92.5	2.0	112.9	2.2
60.0	124.0	9.1	108.9	7.4
90.0	150.9	11.1	102.4	7.4
120	169.4	8.3	92.3	4.9
240	281.6	2.7	92.9	1.0
Mean ± S.D.			99.7 ± 9.9	4.2 ± 2.5

N.A., not applicable.
[a] The solvent extraction procedure was used.
[b] Each three 1 ml aliquots were used; the concentration of *cis*-d_2-EODA was 10 nM.

A pooled urine sample containing *cis*-EODA at a basal concentration of 1.14 nM was used to determine accuracy (recovery) and precision of the method in a relevant concentration range, e.g. up to 34 nM added concentration of synthetic *cis*-EODA. Urine samples (each three 1 ml aliquots) were spiked with *cis*-d_2-EODA at a final concentration of 10 nM. Table 2 shows that *cis*-EODA can be accurately and precisely determined in 1 ml aliquots of human urine. Linear regression analysis between measured (*y*) and added (*x*) *cis*-EODA concentrations resulted in the regression equation $y = 1.152 + 1.022x$ and $r = 0.997$.

Plasma aliquots (each 1 ml) of six healthy volunteers were spiked with *cis*-d_2-EODA at a final concentration of 50 nM, subjected to solvent extraction and derivatized with PFB-Br. The sample were halved. One subset of the samples were further derivatized with BSTFA. After evaporation of solvents and reagents to dryness under a stream of nitrogen gas of the second subset of the samples, the residue was reconstituted in the HPLC eluent (200 μl of acetonitrile–water, 70:30 (v/v)), injected into the HPLC instrument, the HPLC fraction corresponding to the retention time of synthetic *cis*-EODA-PFB ester was collected,

Table 2
Data on the validation of the method[a] for *cis*-EODA in human urine[b]

cis-EODA added (nM)	Measured		Recovery (%)	Precision (R.S.D., %)
	Mean	S.D.		
0.00	1.137	0.031	N.A.	2.7
1.70	2.913	0.307	104.5	10.5
4.25	6.367	0.273	123.1	4.3
8.50	10.68	0.310	112.2	2.9
12.75	13.79	0.963	99.2	7.0
17.00	18.39	0.345	101.5	1.9
34.00	33.00	0.995	93.7	3.0
Mean ± S.D.			105 ± 10.5	4.6 ± 3.1

N.A., not applicable.
[a] The solvent extraction procedure was used.
[b] Each three 1 ml aliquots were used; the concentration of *cis*-d_2-EODA was 10 nM.

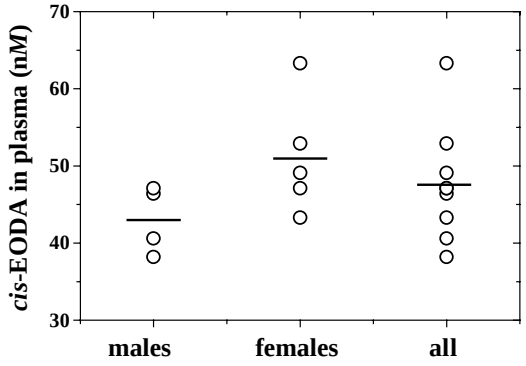

Fig. 5. Basal concentrations of *cis*-EODA in fresh EDTA plasma (1 ml) of four male (23–44 years of age) and five female (23–32 years of age) healthy non-smoking volunteers. *cis*-d_2-EODA was added to plasma at final concentration of 10 nM. SRM of *m/z* 171 from *m/z* 297 and of *m/z* 172 from *m/z* 299. The solvent extraction procedure was used. Samples (1 μl) were injected in the PTV mode. The contribution of exogenous *cis*-EODA was not determined.

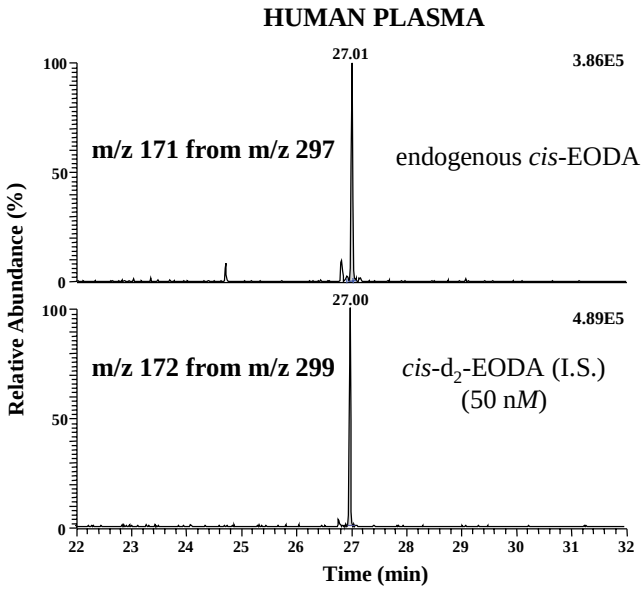

Fig. 6. A representative partial chromatogram from the GC–tandem MS analysis of *cis*-EODA in plasma of a healthy volunteer. Solvent extraction was performed on 1 ml sample aliquot spiked with 50 nM of *cis*-d_2-EODA. SRM of *m/z* 171 from *m/z* 197 for endogenous *cis*-EODA and of *m/z* 172 from *m/z* 299 for the internal standard (I.S.) was performed. The PFB esters of endogenous *cis*-EODA and the internal standard (I.S.) *cis*-d_2-EODA emerged from the column at a retention time of approximately 27 min (underlaid peaks). The concentration of endogenous *cis*-EODA in the plasma sample was determined to be 38.9 nM. Sample (1 μl) was injected in the PTV mode.

compounds were isolated by solvent extraction and derivatized with BSTFA. Quantification of the samples without and with additional HPLC purification by GC–tandem MS yielded almost identical results. The ratio of the concentrations measured without HPLC to those measured with HPLC was determined to be 1.024 ± 0.053 (R.S.D., 5.2%). This finding suggests that no other compounds from plasma interfere with the GC–tandem MS measurement of *cis*-EODA in the method involving a single solvent extraction.

3.4. Basal plasma levels of cis-EODA in healthy humans

cis-EODA was quantified in plasma of freshly obtained blood from healthy, young, non-smoking volunteers. *cis*-EODA was detected in plasma of all volunteers (Fig. 5). Slightly, statistically insignificant higher levels were measured in the female subpopulation (51.1 nM) in comparison with the male subpopulation (43.1 nM; $P = 0.10403$, *t*-test). In urine from spontaneous micturition by these volunteers, *cis*-EODA was measured at 4.1 ± 2.7 nM in males and 5.3 ± 2.3 nM in females (see also below). A typical chromatogram from the GC–tandem MS analysis of *cis*-EODA in human plasma is shown in Fig. 6. The peak eluting in front of *cis*-EODA and *cis*-d_2-EODA co-eluted wit the PFB ester of *trans*-EODA. GC–tandem MS chromatograms obtained without sample purification by HPLC show additional peaks including that of *trans*-EODA-PFB ester (data not shown).

3.5. cis-EODA as a contaminant in laboratory materials

Eight aliquots of potassium phosphate buffer (50 mM, pH 7.4; 1 ml) were spiked with *cis*-d_2-EODA (each 5 nM), samples were subjected to SPE as described for plasma, derivatized and analyzed by GC–tandem MS. These analyses revealed a *cis*-EODA concentration of 2.51 ± 1.29 nM by using Chromabond-LV C18ec columns which are made of polypropylene, but only 0.05 ± 0.01 nM ($n = 3$) by using Chromabond C18ec glass columns. The experiment was repeated by replacing the buffer with water from three different sources, i.e. water prepared in the institute by using a Millipore apparatus, and water from commercial sources, i.e. Ampuwa water (Fresenius; Bad Homburg, Germany) and water of HPLC quality (Baker). GC–tandem MS analysis yielded *cis*-EODA concentrations of 1.46 ± 0.29 ($n = 4$), 0.27 ± 0.09 ($n = 3$) and 0.14 ± 0.02 nM ($n = 2$), respectively.

3.6. Extraction procedure for the simultaneous analysis of cis-EODA and 15(S)-8-iso-PGF$_{2\alpha}$

Because of the potential utility of *cis*-EODA as an indicator of oxidative stress, we were interested in a method allowing the assessment in a single sample of *cis*-EODA and of an established biomarker of oxidative stress, i.e. 15(*S*)-8-*iso*-$PGF_{2\alpha}$, the most prominent member of the F_2-isoprostane family [22]. We have recently shown that 15(*S*)-8-*iso*-$PGF_{2\alpha}$ is best assessed in urine and plasma by GC–tandem MS analysis after IAC extraction [23]. Expectedly, *cis*-EODA will not bind to the antibodies raised against 15(*S*)-8-*iso*-$PGF_{2\alpha}$, so that *cis*-EODA will be easily separated from 15(*S*)-8-*iso*-$PGF_{2\alpha}$. *cis*-EODA will pass through the IAC column, while 15(*S*)-8-*iso*-$PGF_{2\alpha}$ will

selectively bind. For this purpose the following extraction procedure was developed.

Eight different plasma samples (2 ml each) were spiked with *cis*-d_2-EODA (10 nM) and divided into two 1-ml aliquots. One subset was subjected to solvent extraction as described above for plasma *cis*-EODA. The second subset was applied to 4 ml IAC columns. Columns were washed with 2 ml of column buffer (0.1 M potassium phosphate buffer, pH 7.4; 7.7 mM NaN_3; 0.5 M NaCl) and 2 ml of distilled water. These fractions were combined, acidified and subjected to SPE by using the Chromabond-LV C18ec columns as described above for plasma *cis*-EODA. Elution and regeneration of the IAC columns were performed as described elsewhere [23]. Both extraction procedures resulted in very similar results. Linear regression analysis between the *cis*-EODA values (ranging between 46 and 230 nM) obtained from the procedure involving use of IAC columns (y) and those from the solvent extraction procedure (x) resulted in a straight line with the regression equation $y = 0.173 + 0.919x$ and $r = 0.999$. Division on a individual basis of the concentrations obtained from the solvent extraction procedure to those from the use of IAC column extraction yielded a ratio of 1.088 ± 0.023 (R.S.D., 2.1%), indicating the good agreement between the methods.

4. Discussion

The consistent work by Ulsaker and Teien revealed *cis*-9,10-epoxyoctadecanoic acid (*cis*-EODA) as an endogenous constituent in human blood [10,11] and urine [12]. Accurate and precise levels for circulating and excretory *cis*-EODA in humans were, however, not available so far. Ulsaker and Teien estimated by GC–MS the concentration of *cis*-EODA in human urine to be 2.1 nM, i.e. of the same order of magnitude as that of epoxyeicosatrienoic acids [13]. Ulsaker and Teien's investigations also showed that *cis*-EODA is present in fairly substantial amounts in commercially available laboratory plastic ware such as polyvinyl chloride (PVC) [10], in which human blood is collected, and plasma and serum are regularly stored until analysis. The present study confirms the presence of *cis*-EODA in plasma and urine of healthy humans as well as in plastic laboratory material made of polypropylene or polyethylene. Moreover, the present work presents accurately determined *cis*-EODA levels in human plasma.

We synthesized dideuterated *cis*-EODA, i.e. *cis*-d_2-EODA, starting from commercially available dideuterated oleic acid, and used it as an internal standard in a GC–tandem MS method. In developing this method, special attention was paid to the potential contribution of plastic laboratory materials to endogenous *cis*-EODA. Our study shows that exogenous *cis*-EODA contributes by approximately 2 nM to endogenous *cis*-EODA both in plasma and in urine when the solvent extraction procedure or the SPE procedure by using columns made of polypropylene are used. This contribution can be considerably reduced by taking special cautions, e.g. by using glass ware including SPE columns. In plasma (1 ml) of healthy young volunteers we measured *cis*-EODA at a mean level of approximately 48 nM. Thus, exogenous *cis*-EODA contributes to endogenous circulating *cis*-EODA by $\leq$4%. In urine (1 ml) of the same volunteers we measured *cis*-EODA at 4.5 nM, with exogenous *cis*-EODA contributing to urinary *cis*-EODA by approximately 50%. From this point of view plasma rather than urine seems to be the more appropriate analytical matrix for *cis*-EODA.

The GC–MS method of Ulsaker and Teien for the analysis of *cis*-EODA is based upon the reduction of the epoxy and carboxy groups of the molecule to a mixture of 1,9- and 1,10-dihydroxyoctadecane by $LiAlH_4$ [10–12]. Emken has shown that the methyl esters of *cis*- and *trans*-EODA can be analyzed and separated each other in their intact form by GC [24]. Recently, we showed that the pentafluorobenzyl (PFB) esters of intact *cis*- and *trans*-EODA can also be analyzed and separated each other by GC–MS [19]. The PFB esters of *cis*- and *trans*-EODA show virtually identical mass spectra both in the NICI and in the EI mode, but they emerge at different retention times from the GC capillary column. Analogous to other oxidized fatty acids, the possibility of analyzing intact *cis*-EODA as its PFB ester by GC–MS in the NICI mode allow for a considerably more sensitive quantification than the GC–MS analysis of its methyl ester or of the trimethylsilyl ether derivatives of 1,9- and 1,10-octadecanediols in the EI mode [25].

In the NICI mode, the PFB esters of *cis*-EODA and *cis*-d_2-EODA ionize to produce mainly each two intense ions with m/z 297 and 279 for *cis*-EODA and m/z 299 and 281 for *cis*-d_2-EODA (Fig. 7, upper reaction). The appearance in the NICI mass spectrum of *cis*-d_2-EODA PFB ester of an intense ion with m/z of 281 but not an ion with m/z 279, suggests loss of one H_2O molecule but not of one D_2O molecule from the carboxylate anion. A possible explanation for this finding could be that the O atom of the epoxy group leaves the molecule together with two H atoms from the methylene groups at C7/C8 or C11/C12 to produce the anions of the 7,9- and 9,11-octadecadienoic acids. Interestingly, this mechanism seems also to take place under CAD conditions. In addition, collision of the carboxylate anions of *cis*-EODA and *cis*-d_2-EODA with argon atoms in the collision chamber of the mass spectrometer results in cleavage of the molecules between C9 and C10. This could proceed by two different dissociation mechanisms (Fig. 7, reactions A and B, lower panel). Mechanism A produces the anions at m/z 171 (from unlabelled *cis*-EODA) and 172, which still carry the epoxide O atoms in an aldehyde function. On the other hand, mechanism B leads most likely to the formation of the carbene anions with m/z 155 (from unlabelled *cis*-EODA) and m/z 156. These mechanisms seem also to occur in the ion source of the mass spectrometer during the NICI process (see Fig. 2). In mass spectrometry, in its many forms, charged carbenes, notably cumulene carbenes, are

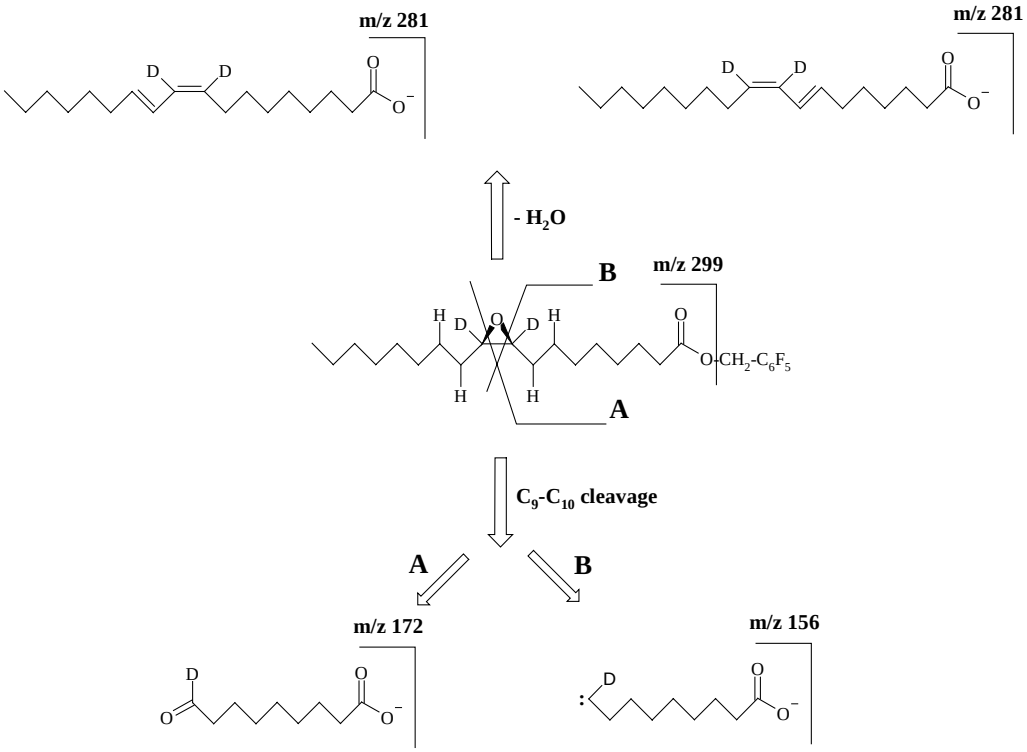

Fig. 7. Proposal of the possible mechanisms leading to the formation of the ions with *m*/*z* of 281, 172 and 156 of the pentafluorobenzyl ester of *cis*-d_2-EODA under negative-ion chemical ionization conditions: *m*/*z*: mass-to-charge ratio; *m*/*z* 299 corresponds to the carboxylate anion of *cis*-d_2-EODA. More details are reported in text.

frequently generated in the gas phase [26]. Quantification of *cis*-EODA in human plasma by SRM of the product ions at *m*/*z* 155 and 156 was as accurate as that by SRM of the product ions at *m*/*z* 171 and 172. However, because SRM of *m*/*z* 171 and 172 allows for a more sensitive detection and also because these ions contain the epoxide O atoms, their detection should result in more accurate and more specific quantification of *cis*-EODA in biological fluids poor in this epoxide, e.g. in human urine.

The direct and unequivocal identification of *cis*-EODA in human plasma and human urine shown in the present study strongly suggests that epoxidation is a physiological metabolic pathway of oleic acid. The origin of circulating and excretory *cis*-EODA as well as the underlying mechanisms are still unknown and remain to be investigated. In vitro studies showed that *cis*-EODA can be produced from oleic acid enzymically by the action of the CYP system [19], with the CYP isoforms 2C2 and 2CAA being implicated [20], as well as non-enzymically, i.e. by lipid peroxidation of oleic acid esterified to lipids [14–16]. Racemic *cis*-EODA has been isolated from total lipids of human leukocytes [17], suggesting a non-enzymic formation of *cis*-EODA. Alternatively, nutrition could be additional source for circulating and excretory *cis*-EODA in humans, as plants are capable of synthesizing enzymically this epoxide from oleic acid [27,28]. Therefore, *cis*-EODA in human plasma may originate simultaneously from different sources, with the contribution of each source being still unknown. This fact may make more difficult the search for the origin and the physiological role(s) of *cis*-EODA. The analytical method described in this article should be useful in forthcoming studies on this issue.

5. Conclusions

cis-EODA can be analyzed by GC in its intact form as methyl and PFB esters due to sufficient thermal stability of the epoxy group, with the PFB esters allowing for a more sensitive detection in the NICI mode. *cis*-d_2-EODA can be easily synthesized by chemical epoxidation of commercially available *cis*-d_2-oleic acid and is suited as internal standard for the quantification of *cis*-EODA in biological fluids by GC–tandem MS. Synthetic laboratory material contains considerable amounts of *cis*-EODA. The contribution of exogenous *cis*-EODA to physiological *cis*-EODA must be considered and can be minimized by using glass ware instead of plastic materials. Plasma seems to be the more appropriate biological matrix for assessing in vivo synthesis of *cis*-EODA. The physiological significance of *cis*-EODA remains to be investigated.

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