Published in final edited form as:

Free Radic Biol Med. 2019 October ; 142: 107–112. doi:10.1016/j.freeradbiomed.2019.02.024.

Adrenic acid non-enzymatic peroxidation products in biofluids of moderate preterm infants

Ángel Sánchez-Illana^a, Vidhi Shah^b, José David Piñeiro-Ramos^a, Juliann M. Di Fiore^{b,c}, Guillermo Quintás^{d,e}, Thomas M. Raffay^{b,c}, Peter M. MacFarlane^{b,c}, Richard J. Martin^{b,c}, Julia Kuligowski^{a,*}

^aNeonatal Research Unit, Health Research Institute La Fe, Avda Fernando Abril Martorell 106, 46026, Valencia, Spain

^bDepartment of Pediatrics, Division of Neonatology, Rainbow Babies and Children's Hospital, Cleveland, OH, USA

^cCase Western Reserve University, Cleveland, OH, USA

^dHealth and Biomedicine, Leitat Technological Center, Avda Fernando Abril Martorell 106, 46026, Valencia, Spain

^eAnalytical Unit, Health Research Institute Hospital La Fe, Avda Fernando Abril Martorell 106, 46026, Valencia, Spain

Abstract

Oxidative stress plays an essential role in processes of signaling and damage to biomolecules during early perinatal life. Isoprostanooids and isofuranooids from the free radical-catalyzed peroxidation of polyunsaturated fatty acids (PUFAs) are widely recognized as reliable biomarkers of oxidative stress. However, their quantification is not straightforward due to high structural similarity of the compounds formed. In this work, a semi-quantitative method for the analysis of adrenic acid (AdA, C22:4 n-6) non-enzymatic peroxidation products (i.e. dihomio-isoprostanes and dihomio-isofurans) was developed. The proposed ultra-performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS) method was applied to the analysis of blood plasma and urine from preterm infants providing information about AdA peroxidation.

Keywords

Dihomio-isoprostanes; Dihomio-isofurans; Lipid peroxidation; UPLC-MS/MS; Preterm infants

*Corresponding author.: julia.kuligowski@uv.es (J. Kuligowski).

Declaration of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.freeradbiomed.2019.02.024>.

1. Introduction

The assessment of oxidative stress (OS) in the perinatal period, and particularly in prematurity is widely discussed in this special issue. In both, physiological and pathological conditions, perinatal oxidative status provides highly valuable information and has helped to understand disease mechanisms and establish predictive or prognostic bio-markers.

One of the most studied OS-related reactions in the perinatal field is the free radical-catalyzed peroxidation of polyunsaturated fatty acids (PUFAs) [1–5]. Since the *in-vivo* free radical-catalyzed peroxidation of arachidonic acid (AA, C20:4 n-6) was discovered [6], numerous studies have been performed focusing on the mechanisms and biomedical implications of the formed compounds that were named isoprostanes (IsoPs) and isofurans (IsoFs). These implications have been recently reviewed by Milne et al. [7] including their employment as biomarkers of oxidative stress-related conditions and their biological activity in the cardiovascular system. During the last decade, the study of lipid peroxidation (LPO) has been extended to other PUFAs thereby identifying different families of LPO products (i.e. isoprostanoids and isofuranoids) from docosahexanoic acid (DHA, C22:6 n-3) and adrenic acid (AdA, C22:4 n-6) among others [8–12]. The study of the AA, DHA and AdA peroxidations are particularly of interest in the neonatal field since these PUFAs play an essential role in the physical properties and function of membranes, influencing their packing and fluidity [13]. Moreover, AdA oxidation may be of particular interest during early human development given the high concentration of this PUFA in the retina and cerebral white matter [14].

Ultra-performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS) has proven its effectiveness to provide adequate sensitivity and selectivity for the analysis of isoprostanoids and isofuranoids in biological samples. Due to their clinical relevance, efforts have been made to chemically synthesize [10,16–18] different isomers of isoprostanoids and isofuranoids [10,12,15] for their use as analytical standards for quantification purposes. However, a comprehensive quantification of the complete set of potentially hundreds of isomers is complex because of the lack of analytical standards and the difficult resolution in the MS and chromatographic dimensions [10,18–20].

To circumvent these limitations, the semiquantitative analysis of IsoPs, IsoFs, neuroprostanes (NeuroPs) and neurofurans (NeuroFs) has been proposed recently [1,21–23]. In this approach, “total parameters” of IsoPs, IsoFs, NeuroPs, and NeuroFs are defined as the total relative response of LPO products that may be detected from the corresponding PUFA *in-vitro* lipid peroxidation at fixed conditions [1]. These conditions include the free radical generator and its concentration, the incubation time, the PUFA concentration, and the preprocessing performed to concentrate the generated compounds. Therefore, the analysis of the selected relative responses in a biological sample allows a straightforward assessment of the free radical attack to the PUFA.

Total parameters of IsoPs, IsoFs, NeuroPs, and NeuroFs were successfully implemented in a UPLC-MS/MS method that enabled their semiquantitative determination together with the quantification of 28 isoprostanoid isomers [5]. However, to the best of our knowledge, to

date total parameters of dihomom-isoprostanes (dihomo-IsoPs) and dihomom-isofuranes (dihomo-IsoFs) formed by AdA peroxidation have not been studied yet.

This work assesses the potential of a relative quantification approach for total dihomom-IsoP and dihomom-IsoF isomers in biofluids. For method set-up and optimization AdA standards were subjected to an *in-vitro* peroxidation procedure and characterized employing UPLC-MS and UPLC-MS/MS. Detection parameters were optimized in terms of sensitivity and the method was applied to urine and plasma samples from preterm infants.

2. Material and methods

2.1. Standards and reagents

AAPH (2,2'-Azobis [2-methylpropionamidine] dihydrochloride), adrenic acid (AdA, 22:4 n-6), β -glucuronidase type IX-A from *E. coli*, and potassium hydroxide (KOH) were purchased from Sigma Aldrich Química S.A. (Madrid, Spain). Methanol (MeOH), ethanol (EtOH), n-heptane (C₇H₁₆), and acetonitrile (CH₃CN) at LC-MS grade were purchased from J.T. Baker (Phillipsburg, NJ, USA). Formic acid (98% w/w) and ethyl acetate (analytical grade) were from Panreac (Barcelona, Spain). Dulbecco's phosphate buffered saline (PBS) containing 137 mM of NaCl, 2.7 mM of KCl, 10 mM of sodium hydrogen phosphate, and 1.8 mM of potassium dihydrogenphosphate at pH 7.4 was prepared dissolving the pre-weighted tablet from VWR (Radnor, PA, USA) in 100 mL of water. Water was ultrapure H₂O generated on a Milli-Q® integral system from Merck Millipore (Darmstadt, Germany). Deuterated prostaglandin F_{2 α} (PGF_{2 α} -D₄) with purity 98% and deuterated incorporation 99% was purchased from Cayman Chemical Company (Ann Arbor, MI, USA) and employed as internal standard (IS).

2.2. Study population and sample collection

The samples employed to evaluate the method's performance were urine and plasma obtained from moderate preterm infants, defined as gestational age between > 31 and 33 weeks at birth, collected at University Hospitals, Rainbow Babies & Children's Hospital, Cleveland Ohio, USA. Exclusion criteria included infants with congenital anomalies. Institutional review board approval was obtained for this study. Parents provided written consent at time of enrollment.

A set of 75 plasma and 23 urine samples collected during the first 3 weeks of life (Days 7–10 and Days 13–17) were employed. For plasma, 500 μ L of venous or capillary blood was collected in lithium heparin plasma separator tubes (BD Microtainer; Franklin Lakes, NJ, USA) and centrifuged at 1000 g for 10 min at 4 °C to obtain plasma that was then stored at –80 °C until analysis. One milliliter of urine was collected employing sterile cotton pads or a newborn Hollister bag and urine was extracted and stored at –80 °C until analysis. Samples were shipped on dry ice and arrived frozen for analysis.

2.3. AdA in-vitro oxidation

AAPH was employed as a model generator of free radicals following previous works with slight modifications [1,8,24–26]. Eight aliquots of 500 μ L of AdA (4.5 mg/mL) and AAPH

(2.7 mg/mL) suspensions in PBS were incubated in 1.5 mL microcentrifuge tubes at 37 °C with mild shaking in a MKR13 thermoblock from Databis AG (Pforzheim, Germany). After 0, 2, 4, 6, 8, 10, 24, and 30 h, aliquots were withdrawn and stored at –20 °C until further processing. Before analysis, each aliquot was thawed, homogenized on a Vortex S0200 mixer from LabNet (Edison, NJ, USA) for 30 s at maximum speed and diluted 1:9 (v/v) in H₂O:MeOH (85:15, 0.1% (v/v) HCOOH) to 900 µL. Blank samples were prepared following the same procedure in the absence of AdA. Diluted aliquots were centrifuged at 16000 g for 10 min at 4 °C using a Biocen 200r centrifuge from Orto Alresa (Madrid, Spain) and supernatants were extracted by solid phase extraction (SPE) following the procedure described for plasma and urine samples prior to UPLCMS/MS analysis.

2.4. Sample preprocessing

Plasma samples were subjected to basic hydrolysis in order to obtain free isoprostanoids and isofuranoids by saponification from the corresponding esters as described in the literature [4,5,12,19]. A volume of 100 µL of plasma was thawed on ice and 100 µL of KOH solution at 15% (w/v) were added. The mixture was incubated at 40 °C for 30 min. For urine samples, the glucuronide conjugates were hydrolyzed using β-glucuronidase from *E. coli* to deconjugate glucuronides of isoprostanoids [27]. One hundred units of β-glucuronidase were added to 600 µL of urine and the mixture was incubated at 37 °C for 90 min.

A volume of 3 µL of aqueous IS solution (20 µM) was added to hydrolyzed samples and diluted to 900 µL with H₂O:MeOH (85:15, 2.8% (v/v) HCOOH) solution for plasma samples and H₂O:MeOH (85:15, 0.1% (v/v) HCOOH) solution for urine samples. Then, the samples were mixed for 30 s at maximum speed and centrifuged at 16000 g and 4 °C for 10 min. For clean-up and pre-concentration of the samples, an SPE procedure employing Discovery® DSC-18 SPE 96-well plates from Sigma Aldrich Química S.A. (Madrid, Spain) was applied following a protocol previously developed for the analysis of lipid peroxidation products [1,2,4,5]. Briefly, first the stationary phase was equilibrated with 1 mL of MeOH and 1 mL of water. Then, the supernatant of the centrifuged sample (approximately 900 µL) was loaded followed by washing with 1 mL of H₂O. Finally, the samples were eluted with 100 µL four times with ethyl acetate. The eluate was evaporated using a miVac centrifugal vacuum concentrator from Genevac LTD (Ipswich, UK) and dissolved in 60 µL solution of H₂O (0.1% v/v HCOOH, pH 3):CH₃OH (85:15 v/v).

2.5. UPLC-MS/MS analysis of dihom-IsoPs and dihom-IsoFs total parameters

An Acquity-Xevo TQS system from Waters (Milford, MA, USA) using negative electrospray ionization (ESI[–]) was employed for UPLC-MS/MS analysis. Chromatographic separation and ESI interface conditions were selected according to previously published methods [1,2,4,5]. A Waters BEH C₁₈ reversed phase column (2.1 × 100 mm, 1.7 µm) was used. Flow rate, column temperature and injection volume were set at 450 µL min^{–1}, 45 °C and 9 µL, respectively. A binary mobile phase H₂O (0.1% v/v HCOOH):CH₃CN (0.1% v/v HCOOH) gradient with a total runtime of 7.0 min was run as follows: from 0.0 to 0.1 min 15% v/v CH₃CN (0.1% v/v HCOOH) (i.e. mobile phase channel B); from 0.1 to 5.0 min %B increased up to 40%; from 5.0 to 6.0 min %B increased up to 75%; between 6.0 and 6.15 conditions were held constant at 75% B followed by the return to initial conditions (i.e. 15%

B) between 6.15 and 6.25 min; conditions were maintained for 0.75 min for system reequilibration. ESI interface conditions were selected as follows: capillary voltage was set to 2.9 kW; source and desolvation temperatures were 150 °C and 395 °C respectively; and nitrogen cone and desolvation gas flows were 150 and 800 L h⁻¹.

In order to determine the chromatographic profile of the *in-vitro* formation of dihom-IsoPs and dihom-IsoFs, the processed AdA oxidized samples (*i.e.* blank sample, 0, 2, 4, 6, 8, 10, 24, and 30 h) were injected into the UPLC-MS/MS system and detected in Selected Ion Monitoring (SIM) mode employing previously reported *m/z* ratios of parent ions for dihom-IsoP and dihom-IsoF isomer detection [5]. Once chromatographic profiles and parent ions of total parameters were established, the processed AdA oxidized samples were detected in daughter ion scan (*i.e.* product ion scan) mode (cone voltage: 30 V, collision energy: 30 eV, mass range: 105–397). The Collision Ion Dissociation (CID) patterns obtained were analyzed and the most intense product ions were identified for each total parameter. Thereafter, collision energy and cone voltages were optimized testing different combinations in a Multiple Reaction Monitoring (MRM) experiment. The MRM transition with the highest intensity after the optimization was selected for each total parameter. Extracts of plasma and urine samples were analyzed employing the parameters shown in Table 1. MassLynx™4.1 from Waters (Milford, MA, USA) was used for data acquisition and processing. Further data analysis was performed in Matlab R2017a (MathWorks, Natick, MA, USA).

3. Results and discussion

3.1. UPLC-MS/MS characterization of AdA non-enzymatic peroxidation products

Dihomo-IsoPs and dihom-IsoFs formed by free-radical oxidation of AdA is a set of compounds with a vast number of isomers extensively studied in the literature [8,10,11,18,28]. They show similar chemical structures and only some of these isomers can be resolved chromatographically resulting in multiple and overlapping peaks. Furthermore, only a limited number of pure analytical standards for the quantitation of those isomers is commercially available or has been described in the literature [10,18–20]. It is also unclear to what extent of knowledge on the augmentation of levels of specific isomers adds to the final interpretation in clinical studies [12]. In this work, an alternative top-down approach for the semi-quantitative measurement of total dihom-IsoP and dihom-IsoF isomers is presented. This approach potentially allows a straightforward determination of dihom-IsoP and dihom-IsoF substance classes in biological samples for their application as biomarkers in the clinical environment.

During analytical method development, the formation of AdA-derived lipid peroxidation products by free-radical attack in an *in-vitro* experiment with different reaction times was characterized by UPLC-MS/MS. For this purpose, the ions [C₂₂H₃₈O₅ – H][–] (*m/z* = 381) and [C₂₂H₃₈O₆ – H][–] (*m/z* = 397) were monitored for dihom-IsoPs and dihom-IsoFs, respectively, taking into account the common molecular formulae of these compounds illustrated in Fig. 2 [8,18,28]. The chromatographic elution window for each total parameter under the employed chromatographic settings was assessed examining the SIM chromatograms of the parent ions for dihom-IsoPs (*m/z* = 381) and dihom-IsoFs (*m/z* =

397) as shown in Fig. 1. For dihom-IsoPs the intensities of the peaks between 5.3 and 6.5 min increased with incubation time. Likewise, for dihom-IsoFs an increase was observed with a slight shift of the elution window between 3.5 and 6.5 min. The higher predicted logP for dihom-IsoPs (i.e. 0.7 more than dihom-IsoFs) [29] might explain the difference in the observed retention time windows. The above-mentioned ranges were established as elution windows for total dihom-IsoPs and dihom-IsoFs, respectively.

The oxidized AdA samples were re-injected in daughter ion scan mode to obtain CID patterns at different reaction times. The combined spectra of the scans acquired during the corresponding elution windows for each total parameter are shown in Fig. 1. Regarding dihom-IsoP product ions, the most abundant m/z were 139, 143, 157, and 155, while in the case of dihom-IsoFs 139, 143, 155, and 183 m/z were the most intense product ions. For optimum sensitivity, different collision energies and cone voltages were tested (see Supplemental Information Figs. S1 and S2). In general, the collision energy had a great impact on the sensitivity, while the cone voltage showed to be less critical. For all values tested, the most sensitive MRM transitions (i.e. the pairs parent > daughter) were 381 > 143 for dihom-IsoPs and 397 > 155 for dihom-IsoFs. The selected transitions for both total parameters have been assigned to the 7-series dihom-IsoPs [8] and 17-series dihom-IsoFs [18] and have been employed previously for the quantification of certain dihom-IsoP and dihom-IsoF isomers [5,10,12,19,20,28]. The fragmentation patterns suggest a charge remote ion mechanism with previous 1 [3]-sigmatropic shift for the 7-series dihom-IsoPs [30,31]. The structures with the proposed fragments are illustrated in Fig. 2. The optimum MS detection conditions selected for the determination of total dihom-IsoPs and dihom-IsoFs based on the analysis of *in-vitro* oxidized AdA are shown in Table 1.

3.2. Total dihom-IsoPs and dihom-IsoFs in urine and plasma samples

A total of 75 plasma and 23 urine samples from preterm infants were analyzed by UPLC-MS/MS employing the parameters of the experimental conditions selected for dihom-IsoP and dihom-IsoF. Chromatographic profiles of representative samples for each matrix are plotted in Fig. 3 together with an *in-vitro* oxidized AdA standard solution and a blank. In urine samples, several peaks were observed at the same retention time and resolution as *in-vitro* oxidation products (i.e. peaks at $\approx$ 5.8, 6.1 and 6.2 min for dihom-IsoPs and $\approx$ 5.5 and 5.75 for dihom-IsoFs). Furthermore, additional peaks appear partially overlapped or at different retention times. In the case of plasma samples, no distinguishable dihom-IsoP peaks were observed. In contrast, for dihom-IsoFs two poorly resolved peaks at $\approx$ 5.5 min were measured.

The area of the entire chromatographic elution window, as determined during the analysis of oxidized AdA standards for each total parameter was selected as a response variable and the limit of quantification (LOQ) was defined as 10 times the blank area. With this cut-off value, both total parameters were detected in most of the urine samples (70%). However, for plasma samples only dihom-IsoFs were detected in a small number of samples. The precision of the determinations was evaluated by the Relative Standard Deviation (RSD) of technical replicates of QC samples (i.e. pooled study samples) and satisfactory values 10%

were obtained. The percentages of samples above the LOQ ($> \text{LOQ}$) and the precision measurements are shown in Table 2.

In order to compensate signal fluctuations originating from instrumental noise and sample treatment, the areas detected in samples $> \text{LOQ}$ were normalized by the area of the IS resulting in relative responses. The obtained relative responses given in procedure defined units (p.d.u.) allow a comparison of results between different batches of samples. Fig. 4 shows boxplots of relative responses obtained for both total parameters detected in blanks, urine and plasma samples. In urine samples, the dihom-IsoPs mean relative response was 16 times higher than that found in the blanks. For dihom-IsoFs, the mean relative response was 13 and 90 times higher than for blanks in plasma and urine samples, respectively. Urine samples showed a relatively large interquartile range compared to plasma samples. The fact that dihom-IsoPs were $< \text{LOQ}$ in plasma samples, while they could be detected in the majority of urine samples might be an indicator for the dynamics of dihom-IsoP formation/excretion as it suggests that the maximum plasma concentration formed from oxidation of AdA remains below the LOQ for the experimental methods used, whereas, dihom-IsoPs were detected in excreted urine from the same infants. Conversely, dihom-IsoFs showed a different profile: they were formed after dihom-IsoPs at sustained oxygen tensions [10,18], and hence they were still present in plasma at the time of sampling and were excreted at higher concentrations in a similar way as described for NeuroFs [1]. This observation is important for the design of future clinical study protocols that aim at targeting those biomarkers in biofluids.

The developed method allows the simultaneous relative determination of the total sum of 7-series dihom-IsoPs and 17-series dihom-IsoFs. In urine, this method proved sufficient sensitivity for detecting relevant concentrations in clinical samples, whereas sensitivity in plasma samples should be improved in order to enable detection of dihom-IsoPs in the majority of samples. This could potentially be achieved by increasing the pre-concentration factor by using higher sample volumes or by modifying the hydrolyzation procedure. It should be noted that the basic/enzymatic hydrolysis employed during the preprocessing of biofluids allowed to detect the sum of free and esterified/glucuronide conjugated forms, however, this analytical method could be applied omitting the hydrolysis step in order to evaluate free forms independently. For clinical validation of the study parameters a larger study population needs to be tested and different sample collection time points are needed for a comprehensive assessment of dynamic concentration ranges. Finally, the sensitivity and selectivity of total dihom-IsoPs and total dihom-IsoFs for detecting brain injury in clinically relevant settings has to be studied.

4. Conclusions

In-vitro peroxidation of AdA was characterized by CID experiments allowing to select the most sensitive and selective MRM transitions for dihom-IsoPs and dihom-IsoFs ($381 > 143$ and $397 > 155$, respectively). The selected measurement conditions were implemented in a UPLC-MS/MS method and applied to urine and plasma samples from preterm infants. Dihom-IsoFs could be detected in both matrices (i.e. urine and plasma), however, dihom-IsoPs were detected exclusively in a limited number (12%) of urine samples. This suggests

that the plasmatic dihom-IsoP concentration was low (i.e. below the LOQ) at the studied sampling time due to the formation and excretion rates. These results enable the design of future studies necessary to establish the accumulation and excretion patterns of total dihom-IsoPs and total dihom-IsoFs and compare them to the obtained regio- or stereoisomer profiles. The usefulness of the total parameters as biomarkers for brain injury will have to be assessed in bigger cohorts of study subjects with well-defined clinical conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors would like to express their gratitude to the parents and their newborns who participated in the study.

Funding

This work was supported by the Instituto Carlos III, Ministry of Economy and Competitiveness, Spain [grant numbers FI16/00380, CP16/00034, and PI17/00127]; the European Society for Paediatric Research [Young Investigator Start-up 2016 grant]; Rainbow Babies and Children's Foundation Fellowship Research Award Program; NIH NICHD Training in Neonatal Research grant [grant number T32-HD060537].

Abbreviations:

AAPH	2,2'-Azobis[2-methylpropionamidine] dihydrochloride
OS	Oxidative stress
LPO	Lipid peroxidation
AA	Arachidonic acid
DHA	Docosahexaenoic acid
AdA	Adrenic acid
IsoPs	Isoprostanes
IsoFs	Isofurans
NeuroPs	Neuroprostanes
NeuroFs	Neurofunans
dihomo-IsoPs	dihomo-isoprostanes
dihomo-IsoFs	dihomo-isofurans

References

- [1]. Kuligowski J, Aguar M, Rook D, Lliso I, Torres-Cuevas I, Escobar J, Quintas G, Brugada M, Sanchez-Illana A, van Goudoever JB, Vento M, Urinary lipid peroxidation byproducts: are they relevant for predicting neonatal morbidity in preterm infants? *Antioxidants Redox Signal.* 23 (2015) 178–184, 10.1089/ars.2015.6262.

- [2]. Kuligowski J, Escobar J, Quintás G, Lliso I, Torres-Cuevas I, Nuñez A, Cubells E, Rook D, van Goudoever JB, Vento M, Analysis of lipid peroxidation biomarkers in extremely low gestational age neonate urines by UPLC-MS/MS, *Anal. Bioanal. Chem* (2014) 1–12, 10.1007/s00216-014-7824-6. [PubMed: 24220758]
- [3]. Cháfer-Pericás C, Rahkonen L, Sánchez-Illana A, Kuligowski J, Torres-Cuevas I, Cernada M, Cubells E, Nuñez-Ramiro A, Andersson S, Vento M, Escobar J, Ultra high performance liquid chromatography coupled to tandem mass spectrometry determination of lipid peroxidation biomarkers in newborn serum samples, *Anal. Chim. Acta* 886 (2015) 214–220, 10.1016/j.aca.2015.06.028. [PubMed: 26320656]
- [4]. Cháfer-Pericás C, Torres-Cuevas I, Sanchez-Illana A, Escobar J, Kuligowski J, Solberg R, Garberg HT, Huun MU, Saugstad OD, Vento M, Development of a reliable analytical method to determine lipid peroxidation biomarkers in newborn plasma samples, *Talanta* 153 (2016) 152–157, 10.1016/j.talanta.2016.03.010. [PubMed: 27130102]
- [5]. Sanchez-Illana A, Thayyil S, Montaldo P, Jenkins D, Quintas G, Oger C, Galano J-M, Vigor C, Durand T, Vento M, Kuligowski J, Novel free-radical mediated lipid peroxidation biomarkers in newborn plasma, *Anal. Chim. Acta* 996 (2017) 88–97, 10.1016/j.aca.2017.09.026. [PubMed: 29137711]
- [6]. Morrow JD, Hill KE, Burk RF, Nammour TM, Badr KF, Roberts LJ 2nd, A series of prostaglandin F₂-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism, *Proc. Natl. Acad. Sci. U.S.A* 87 (1990) 9383–9387. [PubMed: 2123555]
- [7]. Milne GL, Dai Q, Roberts LJ, The isoprostanes—25 years later, *Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids* 1851 (2015) 433–445, 10.1016/j.bbalip.2014.10.007.
- [8]. VanRollins M, Woltjer RL, Yin H, Morrow JD, Montine TJ, F₂-Dihomo-isoprostanes arise from free radical attack on adrenic acid, *J. Lipid Res* 49 (2008) 995–1005, 10.1194/jlr.M700503-JLR200. [PubMed: 18250368]
- [9]. Milne GL, Yin H, Hardy KD, Davies SS, Roberts LJ, Isoprostane generation and function, *Chem. Rev* 111 (2011) 5973–5996, 10.1021/cr200160h. [PubMed: 21848345]
- [10]. Cuyamendous C, de la Torre A, Lee YY, Leung KS, Guy A, Bultel-Poncé V, Galano J-M, Lee JC-Y, Oger C, Durand T, The novelty of phytofurans, isofurans, dihomom-isofurans and neurofurans: Discovery, synthesis and potential application, *Biochimie* 130 (2016) 49–62, 10.1016/j.biochi.2016.08.002. [PubMed: 27519299]
- [11]. Roy J, Le Guennec J-Y, Galano J-M, Thireau J, Bultel-Poncé V, Demion M, Oger C, Lee JC-Y, Durand T, Non-enzymatic cyclic oxygenated metabolites of omega-3 polyunsaturated fatty acid: bioactive drugs? *Biochimie* 120 (2016) 56–61, 10.1016/j.biochi.2015.06.010. [PubMed: 26112019]
- [12]. Galano J-M, Lee YY, Oger C, Vigor C, Vercauteren J, Durand T, Giera M, Lee JC-Y, Isoprostanes, neuroprostanes and phytoprostanes: an overview of 25 years of research in chemistry and biology, *Prog. Lipid Res* 68 (2017) 83–108, 10.1016/j.plipres.2017.09.004. [PubMed: 28923590]
- [13]. Catalá Á, Lipid peroxidation modifies the assembly of biological membranes “The Lipid Whisker Model, *Front. Physiol* 5 (2015), 10.3389/fphys.2014.00520.
- [14]. Martinez M, Tissue levels of polyunsaturated fatty acids during early human development, *J. Pediatr* 120 (1992) S129–S138, 10.1016/S0022-3476(05)81247-8. [PubMed: 1532827]
- [15]. De Felice C, Signorini C, Durand T, Oger C, Guy A, Bultel-Poncé V, Galano J-M, Ciccoli L, Leoncini S, D’Esposito M, Filosa S, Pecorelli A, Valacchi G, Hayek J, F₂ -dihomo-isoprostanes as potential early biomarkers of lipid oxidative damage in Rett syndrome, *J. Lipid Res.* 52 (2011) 2287–2297, 10.1194/jlr.P017798. [PubMed: 21917727]
- [16]. Durand T, Guy A, Henry O, Vidal J-P, Rossi J-C, Rivalta C, Valagussa A, Chiabrando C, Total syntheses of four metabolites of 15-F_{2t}-isoprostane, *Eur. J. Org. Chem* 2001 (2001) 809–819, 10.1002/1099-0690(200102)2001:4<809::AID-EJOC809>3.0.CO;2-6.
- [17]. Galano J-M, Mas E, Barden A, Mori TA, Signorini C, De Felice C, Barrett A, Opere C, Pinot E, Schwedhelm E, Benndorf R, Roy J, Le Guennec J-Y, Oger C, Durand T, Isoprostanes and neuroprostanes: total synthesis, biological activity and biomarkers of oxidative stress in humans, *Prostag. Other Lipid Mediat.* 107 (2013) 95–102, 10.1016/j.prostaglandins.2013.04.003.

- [18]. de La Torre A, Lee YY, Oger C, Sangild PT, Durand T, Lee JC-Y, Galano J-M, Synthesis, Discovery, and quantitation of dihomog-isofurans: biomarkers for in vivo adrenergic acid peroxidation, *Angew. Chem. Int. Ed* 53 (2014) 6249–6252, 10.1002/anie.201402440.
- [19]. Lee YY, Lee JC-Y, LC-MS/MS analysis of lipid oxidation products in blood and tissue samples, *Clin. Metabolomics*, Humana Press, New York, NY, 2018, pp. 83–92., 10.1007/978-1-4939-7592-1_6.
- [20]. Yen H-C, Wei H-J, Lin C-L, Unresolved issues in the analysis of F₂-isoprostanes, F₄-neuroprostanes, isofurans, neurofurans, and F₂-dihomo-isoprostanes in body fluids and tissue using gas chromatography/negative-ion chemical-ionization mass spectrometry, *Free Radic. Res* 49 (2015) 861–880, 10.3109/10715762.2015.1014812. [PubMed: 25812589]
- [21]. Milne GL, Sanchez SC, Musiek ES, Morrow JD, Quantification of F₂-isoprostanes as a biomarker of oxidative stress, *Nat. Protoc* 2 (2007) 221–226, 10.1038/nprot.2006.375. [PubMed: 17401357]
- [22]. Milne GL, Gao B, Terry ES, Zackert WE, Sanchez SC, Measurement of F₂-isoprostanes and isofurans using gas chromatography-mass spectrometry, *Free Radic. Biol. Med* 59 (2013) 36–44, 10.1016/j.freeradbiomed.2012.09.030. [PubMed: 23044261]
- [23]. Song W-L, Lawson JA, Reilly D, Rokach J, Chang C-T, Giasson B, FitzGerald GA, Neurofurans, novel indices of oxidant stress derived from docosahexaenoic acid, *J. Biol. Chem* 283 (2008) 6–16, 10.1074/jbc.M706124200. [PubMed: 17921521]
- [24]. Nourooz-Zadeh J, Halliwell B, änggård EE, Evidence for the formation of F₃-isoprostanes during peroxidation of eicosapentaenoic acid, *Biochem. Biophys. Res. Commun* 236 (1997) 467–472, 10.1006/bbrc.1997.6869. [PubMed: 9240462]
- [25]. Fessel JP, Porter NA, Moore KP, Sheller JR, Roberts LJ, Discovery of lipid peroxidation products formed in vivo with a substituted tetrahydrofuran ring (isofurans) that are favored by increased oxygen tension, *Proc. Natl. Acad. Sci. Unit. States Am* 99 (2002) 16713–16718, 10.1073/pnas.252649099.
- [26]. Song W-L, Lawson JA, Reilly D, Rokach J, Chang C-T, Giasson B, FitzGerald GA, Neurofurans, novel indices of oxidant stress derived from docosahexaenoic acid, *J. Biol. Chem* 283 (2008) 6–16, 10.1074/jbc.M706124200. [PubMed: 17921521]
- [27]. Yan Z, Mas E, Mori TA, Croft KD, Barden AE, A significant proportion of F₂-isoprostanes in human urine are excreted as glucuronide conjugates, *Anal. Biochem* 403 (2010) 126–128, 10.1016/j.ab.2010.04.016. [PubMed: 20406619]
- [28]. Leung KS, Galano JM, Durand T, Lee JC-Y, Current development in non-enzymatic lipid peroxidation products, isoprostanoids and isofuranoids, in novel biological samples, *Free Radic. Res* 49 (2015) 816–826, 10.3109/10715762.2014.960867. [PubMed: 25184341]
- [29]. Chemical Abstracts Service (CAS), SciFinder®. <https://scifinder.cas.org> (Accessed 15 November, 2018).
- [30]. Wheelan P, Zirrollo JA, Murphy RC, Electrospray ionization and low energy tandem mass spectrometry of polyhydroxy unsaturated fatty acids, *J. Am. Soc. Mass Spectrom* 7 (1996) 140–149, 10.1016/1044-0305(95)00628-1. [PubMed: 24203234]
- [31]. Murphy RC, *Tandem Mass Spectrometry of Lipids: Molecular Analysis of Complex Lipids*, Royal Society of Chemistry, 2014.

Fig. 1. SIM (left) and daughter ion scan (right) chromatograms of dihom-IsoPs (top) and dihom-IsoFs (bottom) obtained at different reaction times from AdA.

Fig. 2.
MRM transitions selected for the total parameters and proposed fragmentation mechanisms.

Fig. 3. Chromatograms of total dihomono-IsoPs (left) and dihomono-IsoFs (right). AdA *in-vitro* non-enzymatic peroxidation products after $t = 2$ h and mean $\pm$ standard deviation (thick line and the light shade, respectively) of chromatograms of total parameters measured in 3 replicates of urine samples, plasma samples, and blanks.

Fig. 4. Boxplots of total dihom-IsoPs (left) and dihom-IsoFs (right) for blanks (N = 11), urine (N = 23) and plasma (N = 9) samples. Boxes indicate the 1st and the 3rd quartiles, the median is shown as a blue line, whiskers mark the 9th and 91st percentiles, red circles represent mean concentrations and blue circles are outliers.

Table 1

Mass spectrometric parameters and chromatographic windows selected for the total parameters and the IS (PGF_{2α}-D4). Note: CE stands for collision energy, RT stands for retention time.

Analyte	<i>m/z</i> Parent ion	Cone [V]	CE [eV]	<i>m/z</i> Daughter Ions	Chromatographic RT window [min]
Dihomo-IsoFs	381.00	20	20	143.00	5.3–6.5
Dihomo-IsoFs	397.00	35	25	155.00	3.5–6.5
PGF _{2α} -D4 (IS)	357.00	40	30	197.00	4.97

Table 2

Percentage and number of samples > LOQ as well as RSD in % of QC sample replicates for each matrix and total parameter.

Analyte	Samples > LOQ [%]		RSD QCs [%]	
	Urine (N = 23)	Plasma (N = 75)	Urine (N = 3)	Plasma (N = 3)
Dihomo-IsoPs	70	0	10	-
Dihomo-IsoFs	100	12	4	9