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Relative and absolute reliability of measures of linoleic acid-derived oxylipins in human plasma

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ABSTRACT

Modern analytical techniques allow for the measurement of oxylipins derived from linoleic acid in biological samples. Most validatory work has concerned extraction techniques, repeated analysis of aliquots from the same biological sample, and the influence of external factors such as diet and heparin treatment upon their levels, whereas less is known about the relative and absolute reliability of measurements undertaken on different days. A cohort of nineteen healthy males were used, where samples were taken at the same time of day on two occasions, at least 7 days apart. Relative reliability was assessed using Lin's concordance correlation coefficients (CCC) and intraclass correlation coefficients (ICC). Absolute reliability was assessed by Bland–Altman analyses. Nine linoleic acid oxylipins were investigated. ICC and CCC values ranged from acceptable (0.56 [13-HODE]) to poor (near zero [9(10)- and 12(13)-EpOME]). Bland–Altman limits of agreement were in general quite wide, ranging from ±0.5 (12,13-DiHOME) to ±2 (9(10)-EpOME; $\log_{10}$ scale). It is concluded that relative reliability of linoleic acid-derived oxylipins varies between lipids with compounds such as the HODEs showing better relative reliability than compounds such as the EpOMEs. These differences should be kept in mind when designing and interpreting experiments correlating plasma levels of these lipids with factors such as age, body mass index, rating scales etc.

1. Introduction

The oxylipins are a large family of biologically active oxygenated lipids derived from polyunsaturated fatty acids. Arachidonic acid, for example can be metabolised by cyclooxygenases, lipooxygenases, CYP isoenzymes and by auto-oxidation to produce a wide variety of compounds with different biological properties [1]. Perhaps the best known are the prostanoids, such as prostaglandin (PG) D_2, E_2 and $F_2\alpha$ which mediate a wide variety of biological functions ranging from temperature control, pain sensitivity to kidney function and gastrointestinal barrier protection [1]. However, the other oxylipins are also important: the C_{18} linoleic acid derivatives 9(S)- and 13-hydroxy-10E,12Z-octadecadienoic acid (HODE), for example, are believed to be involved in nociception, possibly by acting as endogenous ligands of the transient receptor potential (vanilloid) 1 receptors [2]. Changed levels of circulating linoleic acid-derived oxylipins have been seen in a variety of disorders including pain states, hyperlipidaemia and diabetes (see [3–8] for examples).

Advances in analytical chemistry have allowed for measurement of a large number of different oxylipins in small volumes of human blood plasma [3,9–13]. This provides a large number of potential uses, both in research and in the clinic. In research, for example, a commonly-used approach is the correlation of levels of given lipids (or combinations thereof), with other measures, such as body mass index, blood pressure, scores in rating scales, etc. In the clinic, it may be possible to use the measured lipid(s) as biomarkers for certain diagnoses, or be supportive in treatment decisions. Such uses, however, require good reliability of the measurements in question, both in terms of the sampling and analysis of the lipids, as well as the natural variability of the levels in the blood. Arachidonic acid-derived oxylipins such as prostaglandins and leukotrienes are a case in point, where artefactual *ex vivo* for-

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mation secondary to enzymatic formation and platelet activation can affect the observed plasma levels, regardless as to how precise the subsequent measurements are [14]. The same is true for oxylipins derived from other long chain fatty acids. Additionally, short-term biological variations can mean that a value taken at one time point is not necessarily representative of the average levels of the lipid in question (see [15] for an example showing variations in plasma concentrations of prostaglandin metabolites in women with premenstrual pain). In the case of lineoleic acid-derived oxylipins, there have been several studies investigating the optimisation of extraction processes, determining the freeze–thaw stability, and the inter- and intra-assay variations of repeated sampling from the same sample and the inadvisability of using heparin as an anticoagulant [3,9–23]. Such studies, crucial though they are, do not consider whether samples taken on different days from the same individuals and under the same conditions show good reliability.

There are a number of methods currently used to establish repeatability and reliability of measurements (review, see [24]). Terminology is something of an issue, but Baumgartner [25] suggested the division into relative reliability and absolute reliability. Relative reliability was defined as being "estimated by using some type of correlation coefficient. It is an indication of the degree to which people maintain their position within a group" [25]. Two useful such measures are the interclass correlation coefficients, ICC [26] and Lin's concordance correlation coefficient, Lin's CCC [27]. Both measures are superior to a simple Pearson's r value, since the latter could give excellent correlation coefficients under conditions where there is an inherent bias in the samples (for example, when the second measurement was always a certain percentage higher than the first measurement) [28,29]. Absolute reliability, on the other hand, gives information concerning the variation of measurements for individuals [24,25]. Bland–Altman's limits of agreement (LoA) [29] as applied to repeated measurements using the same methodology [30] is a commonly used measure of absolute repeatability. It is the difference (in absolute terms when non-transformed data is used, and as a ratio when log transformed data is used, as here) within which 95% of repeated measurements within the same subject would be expected to fall [29,30]. In the contexts described above, a good relative reliability is needed for research studies correlating levels with other parameters, and a good absolute reliability is needed for biomarkers guiding diagnosis/treatment decisions.

In the present study, we have used these methods to assess relative and absolute repeatability of measures of linoleic acid-derived oxylipins in blood plasma samples taken at the same time of day on two occasions from a cohort of healthy non-smoking males.

2. Methods

2.1. Subjects and design

Nineteen healthy non-smoking males (mean age 28 years, range 20–38 years; body mass index 24 kg/m^2, range 19–29 kg/m^2) were investigated on two occasions, between 7 and 46 days apart (mean value 19 days) as part of an as yet unpublished double-blinded, crossover study on the effects of inhaled contaminants on blood oxylipin levels. Subjects were instructed to have an ordinary, light breakfast, but to avoid ham, and also to eat as similarly as possible before the two exposures. They were also asked to refrain from alcohol and caffeine for 24 h pre-exposure and not to use anti-inflammatory drugs or dietary supplements the week preceding the investigations. At 8 a.m. on the day of the experiments, blood samples were drawn by a large bore cannula or butterfly needle and then deposited by needleless syringe into EDTA Vacutainer® tubes where the vacuum had been released. After these samples were collected, the exposure part of the study was undertaken.The samples were collected on ice and centrifuged within 10 min of sampling at +4 °C (2000 × g) and thereafter stored at −80 °C. The samples were placed in the freezer within 5 min after the centrifugation. Blood samples were also taken after the exposures, but the samples described in the present report are the pre-exposure samples alone. The study was approved by the Regional Ethical Review Board in Umeå (Dnr 2012-140-31 M). The procedures were conducted in accordance with the Declaration of Helsinki. All subjects were informed of the purpose and risks of the experiment before the study and gave their written consent.

2.2. Chemicals and reagents

All native and deuterated oxylipin standards and 12-[[(cyclohexylamino) carbonyl]amino]-dodecanoic acid (CUDA) were purchased from Cayman Chemical (Ann Arbor, MI, USA), except 9,10,13-TriHOME and 9,12,13-TriHOME, which were obtained from Larodan (Sweden, Malmö). Methanol (MeOH) and acetonitrile (ACN) were from Merck (Darmstadt, Germany) and isopropanol was from VWR PROLABO (Fontenay-sous-Bois, France). Ethylenediaminetetraacetic acid (EDTA) was purchased from Fluka Analytical, Sigma–Aldrich (Buchs, Switzerland) and butylhydroxytoluene (BHT) was from Cayman Chemical. Acetic acid was from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA) and glycerol was from Fischer Scientific (Loughborough, UK). All solvents and chemicals used were of HPLC grade or higher. A Milli-Q Gradient system (Millipore, Milford, MA, USA) was used to purify water.

2.3. Preparation of calibration curves

Ready-made standard stock solutions or solutions prepared from solid substances were stored in ethanol at −80 °C. The native standard stock solutions were all 10 μg/mL, while solutions of deuterated internal standards were prepared to reach a final concentration of 10 μg/mL (12(13)-EpOME-d$_4$, and 12(13)-DiHOME-d$_4$) and 5 μg/mL (9(S)-HODE-d$_4$, PGE$_2$-d$_4$). Each native stock solution was further diluted with methanol at ten different calibration levels and stored at −80 °C. The deuterated compounds were used as internal standards and added prior to extraction to mimic the isolation of the endogenous compounds from the plasma samples. For each native compound, a suitable internal standard was selected based on structural similarities for quantification purposes. As a verification of the extraction efficiency, recovery rates of each internal standard were calculated by adding a known amount of the recovery standard CUDA in the last step of the procedure. The recovery rates (%, means ± SD, N = 19) of the internal standards were: 12(13)-EpOME-d$_4$, 90 ± 20; 12(13)-DiHOME-d$_4$, 72 ± 11; 9(S)-HODE-d$_4$, 80 ± 6.6; PGE$_2$-d$_4$, 77 ± 9.3.

2.4. Extraction of oxylipins

A previously reported SPE protocol was used for isolation of oxylipins [13]. In brief, oxylipins were extracted using Waters Oasis HLB cartridges (60 mg of sorbent, 30 μm particle size). These were first washed with ethyl acetate and MeOH (2 × 2 mL), then conditioned with 5% MeOH in water (containing 0.1% acetic acid), before loading the plasma sample containing 10 μL IS (50 ng/mL for 12(13)-DiHOME-d$_4$ and 12(13)-EPOME-d$_4$, and 25 ng/mL for 9(S)-HODE-d$_4$, PGE$_2$-d$_4$ and TXB$_2$-d$_4$) and 10 μL antioxidant solution (0.2 mg/mL BHT/EDTA in methanol/water (1:1, V/V)). Oxylipins were eluted using MeOH (3 mL) and ethyl acetate (1 mL) and eluates were evaporated using a MiniVac system (Farmingdale, NY, U.S.A.), reconstituted in 100 μL of MeOH spiked with 10 μL CUDA