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Quantification of the Oxidative Damage Biomarker 2,3-Dinor-8-Isoprostaglandin-F_{2α} in Human Urine Using Liquid Chromatography-Tandem Mass Spectrometry

Haoyue Zhang¹, Dora Il'yasova², Judit Sztaray¹, Sarah P. Young¹, Frances Wang², and David S. Millington^{*,1}

¹Department of Pediatrics, Medical Genetics Division, Duke University Medical Center, Durham, NC

²Cancer Control and Prevention Program, Department of Community and Family Medicine, Duke University Medical Center, Durham, NC

Abstract

F2-isoprostanes are useful biomarkers of oxidative status in humans. We developed an ultra-performance liquid chromatography-tandem mass spectrometric (UPLC-MS/MS) method to quantify 2,3-dinor-8-iso prostaglandin F_{2α}, an urinary metabolite of 8-iso-prostaglandin F_{2α}. Urine was purified by solid phase extraction and analyzed by UPLC-MS/MS with negative ion-electrospray ionization. The method was robust with a mean inaccuracy of 9%, interday and intraday imprecision of 7.5% or lower, and a lower limit of quantification of 0.5 µg/L, equivalent to 0.04 pmol injected onto the column. An analysis time of 6 minutes was shorter than previously published methods and amenable to large studies.

Keywords

Oxidative damage biomarker; UPLC-MS/MS; F2-isoprostanes

Variability in the capacity of antioxidant defense systems to protect against reactive oxygen species (ROS) may contribute to individual differences in susceptibility to chronic diseases [1]. F2-isoprostanes (iPF_{2α}), non-enzymatic products of arachidonic acid peroxidation, are considered sensitive and specific biomarkers of oxidative status. Among them, iPF_{2α}-III (8-iso-PGF_{2α}) and iPF_{2α}-VI have been identified in human urine [2]. Increased iPF_{2α}-III levels have been reported in patients with cardiovascular disease [3], Alzheimer disease [4], and type 2 diabetics [5] and increased iPF_{2α}-VI in patients with Down syndrome [6]. Both were elevated in patients with lung disorders [7] and in heavy smokers [8;9]. 2,3-dinor-8-iso-prostaglandin F_{2α} (2,3-dinor-iPF_{2α}-III) is a metabolite of 8-iso-PGF_{2α} in humans with greater urinary excretion than the parent compound. It is useful as a biomarker for the overall formation of 8-iso-PGF_{2α} and by extension, lipid peroxidation *in vivo*. Traditional analytical approaches for the quantification of isoprostanes include immunological methods (ELISA and RIA) and mass

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*Corresponding Author: DUMC Biochemical Genetics Laboratory, P.O. Box 14991, Durham, North Carolina 27713, United States of America Tel: 919-684-0757 Fax: 919-549-0709 dmilli@duke.edu .

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spectrometry. Immunological methods are simple and inexpensive, but accuracy is difficult to assess due to cross-reactivity with similar endogenous molecules [10]. Gas-chromatography mass spectrometry has high sensitivity and specificity for individual isoprostanes, but is technically demanding, labor intensive and may potentially result in loss of target compounds during extraction and derivatization [3;11]. Recently developed tandem mass spectrometric (MS/MS) methods achieved adequate sensitivity and resolution of isomers without derivatization, but with moderately long run times [12;13;14;15;16]. The objective of this work was to develop a more rapid MS/MS assay amenable to large studies for urinary 2,3-dinor-iPF_{2α}-III utilizing ultra performance liquid chromatography (UPLC).

The internal standard (IS) chosen for this study was a stable isotope-labeled analogue of the parent compound, iPF_{2α}-III-d₄ which is commercially available and affordable. Sample preparation and analysis is straightforward: 500 μL urine was mixed with 20 ng iPF_{2α}-III-d₄ IS (Cayman Chemicals, Ann Arbor, MI), acidified to pH 2-3 using 40 μL 1M HCl, and made up to a final volume of 1 mL with DI water. Samples were vortex-mixed and centrifuged at 15,000g for 3 min to remove suspended material, before loading onto solid phase extraction (SPE) cartridges (Oasis HLB 30 mg, Waters Inc, Milford, MA) primed with methanol and DI water. The cartridges were washed with 1 mL 0.1 M HCl, 1mL MeOH: DI H₂O (5:95, v/v), followed by 1 mL hexane and samples were eluted using 1 mL MeOH. The eluates were dried under N₂ at 40°C, and reconstituted in 150 μL of mobile phase consisting of 90:10 (v/v) solvent A (0.15% NH₄OH in H₂O) and solvent B (0.15% NH₄OH in MeOH:MeCN (5:95,v/v)). Samples were analyzed by UPLC-MS/MS on an Acquity UPLC system and TQD triple quadrupole mass spectrometer (Waters Corporation, Milford, MA) with electrospray ionization in negative ion mode. 5 μL of sample was injected onto an Acquity UPLC™ BEH C18 1.7 μm 2.1× 50 mm column (Waters Corp) heated to 40°C and separated with gradient elution at a flow rate of 400 μL/min. 90:10 (v/v) solvent A: solvent B was changed to 81:19 (v/v) A: B over 5.4 min, returned to 90:10 (v/v) A: B over 0.1 min and equilibrated for a further 0.5 min. The column eluant was directly infused into the mass spectrometer. Mass spectrometer parameters were as follows: capillary voltage 3.6 kV, cone voltage 30V, and collision energy of 13eV for 2,3-dinor-iPF_{2α}-III and 24eV for iPF_{2α}-III-d₄. Two transitions were acquired in selected reaction monitoring (SRM) mode for both the analyte and internal standard. For 2,3-dinor-iPF_{2α}-III, the transitions were m/z 325>237 (primary), and 325> 137 (secondary). For iPF_{2α}-III-d₄, the transitions were m/z 357>197 (primary) and 357> 313 (secondary). These transitions are well documented [15;17]. The signal ratio of the primary transitions was used to quantify the analyte and the secondary transitions were used as purity checks. The peak area ratio of the primary to secondary transition was determined for each specimen and compared with a pre-determined value derived from calibration samples. This ratio remained consistent over time in calibration samples (mean ratio: 10.3±1.51; RSD =15%, n=49, over 18 weeks). Deviation from this ratio would suggest the presence of an interfering compound. SRM chromatograms corresponding to the primary and secondary transitions for 2,3-dinor-iPF_{2α}-III and iPF_{2α}-III-d₄ are shown in Fig 1A (standard mix) and 1B (control urine). The chromatograms of 2,3-dinor-iPF_{2α}-III primary and secondary transitions for a urine with analyte concentration near the limit of quantification, is shown (Fig 2). The resolution using the UPLC™ BEH C18 column is similar to that observed with conventional HPLC C18 columns, but with a significant reduction in analysis time from 20 min [12] to 6 min. 2,3-dinor-iPF_{2α}-III eluted at 1.9 min and the internal standard eluted at 3.8 min. Calibration samples were prepared by mixing 0.25 to 10 ng 2,3-dinor-iPF_{2α}-III standard (Cayman Chemicals, Ann Arbor, MI) prepared in MeOH:DI H₂O (20:80, v/v) with 500 μL of a control urine with low endogenous 2,3-dinor-iPF_{2α}-III. Calibration samples were purified using SPE and analyzed as described above. Calibration curves were derived from the peak area ratio of the primary SRM transition for 2,3-dinor-iPF_{2α}-III standard to that of iPF_{2α}-III-d₄ IS using Targetlynx® software (Waters Corp), with manual correction of integration when needed. The curve was linear over the calibration range 0.5 to 20 μg/L (r²: 0.991 ± 0.005, mean ± SD, n = 9) and the

calibration was stable for at least 7 weeks (slope: 1.39 ± 0.10 ; intercept: 0.19 ± 0.14 , $n = 9$). The mean observed percentage deviation over the calibration range of back-calculated concentrations was between -1.3% and 7.8% with an imprecision of $\leq 13\%$. Accuracy was evaluated in samples prepared from the same control urine with low endogenous 2,3-dinor-iPF_{2α}-III spiked with low ($1.2 \mu\text{g/L}$) and high ($6 \mu\text{g/L}$) concentrations of 2,3-dinor-iPF_{2α}-III standard. Mean inaccuracy was 9% ($n = 5$) for both the low and high concentration. Recovery of 2,3-dinor-iPF_{2α}-III was evaluated using control urine samples fortified with 2, 10 and $20 \mu\text{g/L}$ of 2,3-dinor-iPF_{2α}-III and extracted using the sample preparation procedure described above. 20 ng internal standard was spiked in each of the final extracts. The recovery was determined by comparing the mean response of the fortified control urine samples with the response of the same concentrations of pure standard and 20 ng internal standard prepared in mobile phase and directly injected onto the column. Recoveries were 86% ($\text{RSD} = 14\%$), 90% ($\text{RSD} = 8.0\%$) and 90% ($\text{RSD} = 6.1\%$) for the samples spiked with 2, 10 and $20 \mu\text{g/L}$, respectively ($n = 5$).

Precision of the assay was evaluated by replicate analysis pooled control urines with low ($0.7 \mu\text{g/L}$) and high ($20 \mu\text{g/L}$) endogenous 2,3-dinor-iPF_{2α}-III. Intra-day imprecision (RSD) was 7.5% ($n = 6$) and 5.3% ($n = 7$), and inter-day imprecision was 5.3% and 7.3% ($n = 8$, over eight weeks) for the low and high samples, respectively.

The limit of quantification was defined as the lower limit of calibration ($0.5 \mu\text{g/L}$: equivalent to 0.04 pmol injected onto the column) where accuracy and precision were within acceptable limits ($<20\%$). The limit of detection, defined by a signal to noise ratio of 3 to 1, was 0.01 pmol . The urine samples were stable after three freeze-thaw cycles, in storage at -80°C for at least 6 months and at room temperature for at least 24 hour, as judged by the analysis of 2,3-dinor-iPF_{2α}-III in low and high QC samples.

Using this assay, we investigated intra- and inter-person variation of urinary 2,3-dinor-iPF_{2α}-III. Three urine samples were collected at 4 week intervals from 21 volunteers (11 females, 10 males) aged 25 to 75 years. All participants were consented for the study under a protocol approved by DUMC Institutional Review Board. Urine specimens were stored at -80°C prior to analysis. Statistical analysis was performed using SAS 9.2 (SAS institute, Cary NC). The overall mean $\pm$ SD value of 2,3-dinor-iPF_{2α}-III was $4.3 \pm 0.3 \mu\text{g/g CR}$ and was comparable to literature values [12;13]. The mean 2,3-dinor-iPF_{2α}-III level for females (5.0 ± 0.6) was significantly higher ($p = 0.003$, t-test) than that for males (3.6 ± 0.3). There was no significant difference in mean values for samples collected from the same subject at the different times ($p > 0.6$, paired t-test). The contributions of intra- and inter-person variation to the total variance were 40% and 60% , respectively. Intra- class correlation coefficient was 0.6 . When the analysis was limited to the urinary samples collected in the morning (before 9:00 am, $n = 47$), intra-person variation decreased to 30% and was similar to that observed previously for repeated measures of plasma F₂-isoprostanes (28%) [18]. This suggests standardizing urine collections to first morning voids may decrease intra-person variation.

In conclusion, we have developed a rapid and specific method for the analysis of urinary 2, 3-dinor-iPF_{2α}-III level that is suitable for studies involving larger cohorts than have been studied previously [12;13].

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Abbreviations

MS/MS	Tandem mass spectrometry
UPLC	Ultra performance liquid chromatography
SRM	Selected reaction monitoring
SPE	Solid phase extraction
MeOH	Methanol
MeCN	Acetonitrile
ROS	Reactive oxygen species
8-iso-PGF _{2α}	8-iso-prostaglandin F _{2α}
2, 3-dinor-iPF _{2α} -III	2,3-dinor-8-iso-prostaglandin F _{2α}
μg/g CR	μg per g creatinine
RSD	Relative standard deviation also referred to as CV%
DI	De-ionized

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Figure 1.

Chromatograms of the primary and secondary SRM transitions for (A) 3 ng 2,3-dinor-iPF_{2α}-III (* labeled peak) mixed with 20 ng of iPF_{2α}-III-d₄ internal standard (IS) in 150 μL of mobile phase, (B) control urine spiked with 20 ng IS and a measured endogenous 2,3-dinor-iPF_{2α}-III concentration of 5.2 μg/L.

Figure 2.

Chromatograms of 2,3-dinor-iPF_{2α}-III primary and secondary SRM in control urine spiked with 20 ng IS and a measured endogenous 2,3-dinor-iPF_{2α}-III concentration of 0.51 μg/L.