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*Short Communication***Stability of Measurements of Biomarkers of Oxidative Stress in Blood Over 36 Hours**Tianying Wu,^{1,3} Nader Rifai,³ L. Jackson Roberts II,⁵ Walter C. Willett,^{1,2,4} and Eric B. Rimm^{1,2,4}

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Abstract

Oxidative stress is hypothesized to play an important role in a variety of chronic diseases, but the short-term and long-term stability of measurements of biomarkers related to oxidative stress remains unclear. The objective of this study was to evaluate the stability of measurements of malondialdehyde (MDA), F₂-isoprostanes, and fluorescent oxidation products in blood stored on ice within 36 hours until processing. Whole blood samples from six healthy women were processed at 0, 24, and 36 hours after being stored on ice. MDA was measured by the thiobarbituric acid-reactive substances assay with high-pressure liquid chromatography. F₂-isoprostanes were measured by gas chromatography/mass spectrometry. The fluorescent oxidation products were measured by spectrofluorometry. Measurements of fluorescent oxidation products were very stable up to 36 hours. Intraclass correlation coefficients (ICC) were >0.95 for each time interval (0 to 24 and 0 to 36 hours). Measurements of MDA were the least stable. The median increased significantly from 0 to 24 hours and from 0 to 36 hours. The ICC for MDA for each time interval (0 to 24 and 0 to 36 hours) was <0.1. Finally, the median of F₂-isoprostane measurements at each time point also increased significantly. ICCs were 0.45 for 0 to 24 hours and 0.09 for 0 to 36 hours. We conclude that measurements of fluorescent oxidation products in blood remain stable for up to 36 hours and may be used in large prospective epidemiologic studies of chronic diseases. (Cancer Epidemiol Biomarkers Prev 2004;13(8):1399–402)

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Introduction

Many biomarkers have been developed to evaluate oxidative stress. Malondialdehyde (MDA) and F₂-isoprostanes are two markers widely used. Increased plasma MDA levels (1-4) and F₂-isoprostanes (5-9) have been found in a variety of disease conditions and among smokers. Recently, Mezzetti et al. (10) have shown that fluorescent products of lipid peroxidation (FLIP), many of which are conjugated Schiff bases (11), predict the risk of cardiovascular events in a group of healthy, elderly subjects. Those with FLIPs in the highest quartile had a risk seven times greater than those with FLIPs in the lowest quartile. In addition, these Schiff base products and their derived products—advanced glycation end products (12)—have been implicated in several diseases such as Alzheimer's disease (13, 14), cancer (15), nondiabetic nephropathy (16), and diabetes complica-

tions (17). Although markers of fluorescent oxidation products have not been widely used, this assay is relatively simple and has potential to be used in epidemiologic studies.

The influence of the time to processing on the stability of biomarkers needs to be examined before applying them to epidemiologic studies. In large cohort studies such as the Nurses' Health Study and the Health Professionals Follow-up Study, blood samples are collected, placed on ice, and shipped to the central laboratory overnight or the next day. The time frame for processing the samples after their placement on ice ranges from 0 to 36 hours. To our knowledge, the stability of measurements of MDA, F₂-isoprostanes, and fluorescent oxidation products in whole blood samples stored on ice has not been examined previously.

Materials and Methods

Blood Collection and Processing. Blood samples were collected from six healthy women ages 25 to 45 years. The project was approved by the Harvard School of Public Health Institutional Review Board, and all volunteers gave written informed consent. For each subject, blood was collected into three 10 mL Vacutainer (sodium

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heparin) tubes, which were placed immediately on ice. These three tubes were processed at 0, 24, and 36 hours. Samples processed at 24 and 36 hours were first placed in a Styrofoam container with ice packs, simulating conditions of overnight and next-day mail service in our main cohort study. At each time point, whole blood samples were centrifuged and plasma samples were aliquoted for storage in liquid nitrogen (-140°C). Each sample was aliquoted in duplicate tubes, assigned a different identification number, and randomly placed in the analysis batch with respect to the three different processing time periods. All assays were conducted without knowledge of the status of each sample.

Assays. Measurement of MDA via the thiobarbituric acid (TBA)-reactive substances assay was carried out with high-pressure liquid chromatography, which is considered to be relatively sensitive and more specific than the TBA assay by spectrofluorometry (18). The high-pressure liquid chromatography model was Shimadzu SIL-10A with fluorescent detector (Shimadzu RF-10A) with 532/553 nm wavelength (excitation/emission). We used the procedures described by Young and Trimble (19). The only modification was our use of a different column. The procedures are described briefly below. There were two steps in this assay: derivatization and neutralization. After the neutralization, a 40 μL suspension was injected (automated injector with 50 μL sample loop) onto a C18 column (ODS Ultrasphere, 5 μm , 150 \times 4.6 mm; Beckman Coulter, San Diego, CA) using a mobile phase that contained 50% methanol/50% phosphate buffer at pH 6.5 at a flow rate of 0.8 mL/min. MDA (Sigma-Aldrich, St. Louis, MO) was used as an external standard. MDA standards (0, 0.24, 0.48, 1.2, 2.4, and 4.8 $\mu\text{mol/L}$) were prepared with 1,1,3,3-tetramethoxypropane, which yields equivalent amounts of MDA under the conditions of the reaction. The correlation for the linearity of the standard curve was 0.99. We also have set up a standard curve by spiking the above MDA standards with plasma; the recovery of MDA from spiked plasma ranged from 95% to 100%. Intraassay and interassay coefficients of variation were $<15\%$.

F_2 -isoprostanes were measured by gas chromatography/mass spectrometry as described previously (20-22). Intraassay and interassay coefficients of variation averaged 9%. This assay is well established and used in several studies (5-9, 21). As described in Morrow and Roberts (22), the accuracy of this assay is 96%, which is determined by quantification of an added known amount of the F_2 -isoprostanes to plasma.

The procedures for measuring fluorescent oxidation products were modified from the method of Shimasaki (23). In brief, 0.2 mL of plasma samples was mixed with 1 mL of ethanol/ether (3:1 v/v) in borosilicate glass tubes (1495935c, Fisher, Hanover Park, IL) and vigorously mixed on a vortex mixer. The mixed solution was centrifuged for 10 minutes at 3000 rpm, and 1 mL of supernatant was added to cuvettes for spectrofluorometric readings. The fluorescence can be determined as relative fluorescence intensity units per milliliter of plasma at 360/430 nm wavelength (excitation/emission) by a spectrofluorometer (Shimadzu RF-5301 PC). Quinine sulfate in diluted 0.1 N H_2SO_4 was used for calibration. To verify whether this assay can reflect the change of oxidative insult, we did the following experiments. Pooled plasma samples were

diluted (~ 150 -fold, 0.5 mg/mL protein) and oxidized with 5 $\mu\text{mol/L}$ CuSO_4 at 37°C . An aliquot of plasma was removed after 12, 24, 36, 48, 72, 96, 120, 144, 168, 192, and 216 hours. EDTA (1.5 mg/mL) was added immediately to halt the oxidation reactions. Aliquots at each time point were measured by this assay. We plotted a curve based on the results. The fluorescent intensity range of our sample was well below the range of this curve. Accuracy of analysis was also achieved by inclusion low, medium, and high quality controls; the intraassay and interassay coefficients of variation were $<10\%$. The quality controls were obtained from the serially diluted pooled plasma samples oxidized for 192 hours (as described above).

Statistical Analysis. To assess within-subject coefficients of variation, each sample was aliquoted in duplicate tubes for each subject at each time point and randomly placed in the analysis batch with a dummy ID. However, in large epidemiologic studies where plasma is limited, it is not always possible to conduct two measurements on the same person. Therefore, to be conservative, we only use the first observation for primary analysis (presented in Table 1). However, we also conducted a secondary analysis using the means of the duplicates for each subject to illustrate the marginal increase in accuracy with duplicate measurements (Table 1). If a variable was not normally distributed, log-transformed data were used for statistical analysis. A paired t test was used to compare means between different time points within an individual. Intraclass correlation coefficients (ICC) were calculated by ANOVA to determine the stability over time within an individual (24). All analyses were conducted in SAS 6.12. By definition of the ICC (between-person variance divided by the sum of the between-person and within-person variances), if a biomarker is not stable over time, the within-person variance will increase and the ICC will decrease. Therefore, we also calculated the Pearson (for normally distributed data) and Spearman (for non-normally distributed data) correlations because they are sensitive only to relative stability between subjects.

Results

The coefficients of variation were 8.32%, 15.8%, and 10.2% for the measurement of fluorescent oxidation products, MDA, and F_2 -isoprostanes. These coefficients of variation are all in a reasonable and acceptable range. Table 1 shows the median value and stability of measurements of the three oxidation markers, MDA measured by TBA, F_2 -isoprostanes by gas chromatography/mass spectrometry, and fluorescence intensity of oxidation products at 0, 24, and 36 hours. In our primary analysis based on single measurement, concentrations of MDA increased significantly from 0 to 24 hours and from 0 to 36 hours. Esterified F_2 -isoprostanes also increased significantly from 0 to 36 hours. Fluorescence intensity of fluorescent oxidation products remained unchanged from 0 to 36 hours. As shown in Table 1, the ICCs of the TBA assay were poor for 0 to 24 and 0 to 36 hours. Esterified F_2 -isoprostane had better reproducibility for 0 to 24 hours (ICC = 0.45) and for 0 to 36 hours (ICC = 0.09) than MDA. The ICCs for fluorescent oxidation products

Table 1. Stability of whole blood samples on ice from 0 to 36 hours before processing (n = 6)

	Median (Interquartile Range)			P for Paired t Test		ICC	
	0 Hours	24 Hours	36 Hours	0-24 Hours	0-36 Hours	0-24 Hours	0-36 Hours
<i>Analysis based on single measurement per subject</i>							
MDA ($\mu\text{mol/L}$) via TBA assay	1.94 (1.59-2.50)	2.92 (2.87-3.33)	3.85 (3.03-4.70)	0.03	0.001	-0.09	-0.11
Esterified F ₂ -isoprostanes* (pg/mL)	46.0 (35.0-69.0)	83.0 (76.0-95.0)	129.5 (84.0-183.0)	0.05	0.01	0.45	0.09
Fluorescent assay (fluorescent intensity/mL)	164 (127-190)	163 (134-200)	163 (134-194)	0.3	0.5	0.96	0.98
<i>Analysis based on duplicate measures per subject</i>							
MDA ($\mu\text{mol/L}$) via TBA assay	1.97 (1.53-2.43)	3.21 (2.91-3.54)	3.71 (3.32-4.6)	0.01	0.003	0.16	0.02
Esterified F ₂ -isoprostanes† (pg/mL)	55.3 (47.5-76.5)	73.5 (69.0-88.5)	101.3 (68.3-123.5)	0.04	0.02	0.74	0.51
Fluorescent assay (fluorescent intensity/mL)	177 (129-208)	170 (145-202)	166 (142-198)	0.7	0.3	0.97	0.97

*Data were log transformed.

†Geometric means are presented.

were excellent (0.96 and 0.98 for 0 to 24 and 0 to 36 hours, respectively). Results from Pearson or Spearman correlation also confirmed that fluorescent oxidation products were the most stable. The correlations for fluorescent oxidation products were 0.99 and 0.98 for 0 to 24 and 0 to 36 hours, respectively (P 's < 0.05). The correlations for MDA were 0.39 ($P = 0.4$) and 0.89 ($P = 0.02$) for 0 to 24 and 0 to 36 hours, respectively. The correlations for F₂-isoprostanes were 0.89 ($P = 0.02$) and 0.2 ($P = 0.7$) for 0 to 24 and 0 to 36 hours, respectively.

In addition, we reanalyzed the data using the means of the duplicate assessments for each subject (Table 1). The results were similar, although the ICCs improved modestly for F₂-isoprostanes (0.74 and 0.51 for 0 to 24 and 0 to 36 hours, respectively).

Discussion

In this pilot study, we found that measurements of fluorescent oxidation products by FLIP assay in blood samples held on ice for 36 hours until processing remained essentially unchanged. In contrast, measurements of MDA significantly increased from 0 to 24 hours and from 0 to 36 hours. F₂-isoprostane measurements increased in the same direction as MDA, although the magnitude was less. The reason for the increases in the levels of MDA and F₂-isoprostanes detected in blood during storage is undoubtedly due to autoxidation of plasma lipids with artifactual generation of MDA and isoprostanes. Why such increases were not observed with products measured by the FLIP assay is not clear.

Although the sample size was small ($n = 6$) in this study, the results of FLIP assays were substantially better than those for MDA or F₂-isoprostanes. The coefficients of variation were the lowest for FLIP assay (<10%); the percentage of differences in the median for the time intervals (0 to 24 and 0 to 36 hours) was <6%, and both ICC and Pearson correlation for each time interval were excellent. The results for MDA suggest that this assay cannot be used in epidemiologic studies when samples are not processed immediately. Not only did the median increase from 0 to 24 hours and from 0 to 36 hours, but the ICCs were also poor (<0.1). An ICC of <0.1 indicates

that >90% of the variation in concentration in the 36-hour samples are attributable to the delay in processing.

For F₂-isoprostanes, both ICCs and Spearman correlations were modest or good for 0 to 24 hours but not for 0 to 36 hours. Whether this marker is potentially useful in large epidemiologic studies (with a single sample) is unclear because of the small sample size. For studies with duplicate samples and a processing time of whole blood within 24 hours, F₂-isoprostanes may still be acceptable. F₂-isoprostanes are considered to be one of the best lipid peroxidation markers; thus, further work is warranted to explore the reproducibility and validity of this marker with a larger sample size.

The use of different anticoagulants for blood collection may affect the interpretation of our results. For the TBA assay and FLIP assay, we found little differences between fresh EDTA and heparin blood samples. However, we have not compared these two types of blood collection for the three measurements after whole blood has been kept on ice for up to 36 hours. Because EDTA is a metal chelator, it theoretically should prevent oxidation. Kontush and Beisiegel (25) showed that plasma samples prepared with different anticoagulants exhibit different oxidation kinetics. The oxidation rate of EDTA plasma is lower than that of heparin plasma. However, Pischon et al. (26) found that, after storage of blood on ice for 36 hours, the levels of adiponectin significantly increased in blood collected with EDTA but not in blood collected with heparin. His finding raises concerns about EDTA collection versus heparin collection, although adiponectin is an adipose-derived hormone, not an oxidation marker.

FLIPs may be an insufficient description of these oxidation products. In general, they are carbonyl compounds—aldehydes cross-linked with amino acids (11, 12). Aldehydes can derive from lipids, carbohydrates, amino acids, and DNA (11, 12, 27). Thus, fluorescent oxidation products are markers of overall lipid, protein, carbohydrate, and DNA oxidation. Although the FLIP assay seems to have potential for application in epidemiologic studies, the stability of measurements using the FLIP assay in long-term storage, its reproducibility over time within persons, and its determinants also need to be tested before this assay can be considered useful in epidemiologic studies.

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