Paraoxonase-1 Enzyme Activity Assay for Clinical Samples: Validation and Correlation Studies

Paraoxonase-1 (PON1) enzyme is reported in various types of tissues and linked to numerous pathophysiological disorders. It is a potential biomarker in many pathological conditions such as cardiovascular diseases.

Material/Methods: We conducted several small-scale studies to evaluate PON1 performance as affected by sample types, storage, and interferences. We also carried out short-term studies to compare the performance of the widely used PON1 assay to the similar commercially available PON1 kit assay method; sample size for the method comparison was N=40, and the number varied for other validation experiments.

Results: Our studies using various types of anticoagulants show that samples collected in tubes with NaF, citrate, EDTA, clot activator, and sodium heparin have increased PON1 levels that are 49%, 24.5%, 19.8%, 11.4%, and 8%, respectively, higher compared to serum samples collected in plain tubes. However, samples collected in lithium heparin tubes demonstrated 10.4% lower PON1 levels compared to serum collected in plain tubes. Biological interference such as hemolysis has little effect on PON1 levels; however, samples spiked with lipids have shown 13% lower PON 1 levels. Our studies comparing the PON1 method commonly available for PON1 assay and a similar non-ELISA commercially available PON1 kit method showed a weak Spearman correlation coefficient of \( R^2 = 0.40 \) for the range of 104.9–245.6 U/L.

Conclusions: The current study provides new validation data on enzyme PON1 performance. While no appreciable change was seen with storage, samples type affects the enzyme performance. Our results should encourage additional clinical studies to investigate other aspects of factors known to affect PON1 enzyme function and performance.

MeSH Keywords: Biochemical Processes • Cardiovascular Diseases • Enzyme Activation

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**Background**

Paraoxonases (EC 3.1.8 Phosphoric-triester hydrolases) are a family of enzymes originally known for their ability to hydrolyze exogenous toxic organophosphate compounds such as the insecticide paraaxon. Studies have shown that the enzyme exists as Paraoxonase 1 (PON1), Paraoxonase 2 (PON2), and Paraoxonase 3 (PON3), encoded by 3 separate genes on the same chromosome 7 in humans [1–3]. These enzymes catalyze a wide range of substrates of biological importance such as organophosphorus compounds (paraoxon), including esters of phosphonic and phosphinic acids. The enzyme is inhibited by chelating agents. Several compounds such as PD-116122, PD-65950, PD-92770, and PD-113487 are described in the literature as inhibiting the enzyme activities. PON1 requires divalent cations for its activity. The diverse nature of its action has made the study of its mechanism (s) of action of great interest for basic and clinical use [4–6]. PON1 enzyme is mainly expressed in the liver, but its expression is also reported in various types of tissues and is linked to numerous pathophysiological disorders. It is a potential biomarker for many pathological conditions such as cardiovascular diseases, neurological disorders, and liver diseases. Results of in vitro and in vivo studies have shown that PON1 has antioxidant properties that are closely associated with (HDL) ApoA1 protein. We and others have shown that bioactive molecules such as dietary polyphenols, aspirin, and its byproduct, salicylate, stimulate PON1 activation in mouse liver and HepG2 cells [7–10]. Studies on its activity, function, and genetic makeup have revealed an anti-oxidative stress protective role for PON1. PON1 inhibits copper-induced HDL oxidation by prolonging oxidation lag phase, and reduces peroxide and aldehyde content in oxidized HDL [11].

Although PON1 assay can provide substantial diagnostic value, the assay has only been used for investigative research, particularly in clinical longitudinal studies [1,2]. The reason for the constraints in its use can be attributed to the lack of a validated and robust assay beyond the current research methods. The fact that the substrate commonly used for PON1 measurement is toxic and unstable, in addition to the lack of an automated method, has also contributed to the inability to use it clinically. Assay performance is affected by factors such as length of sample storage, temperature, effect of the use of anticoagulants (sample type), quality of the samples (particularly in presence of interferences induced by lipids or hemolysis), variability of PON1 measurements (especially in longitudinal studies), and the effect of drugs intake; these are either poorly understood or completely missing. The current study was conducted to investigate and address some of these discrepancies; emphasizing on the role of sample types, storage, and interferences in PON1 assay performances. In addition, we carried out limited studies to compare the performance of the PON1 assay, widely used in research studies, to a similar method using the commercially available PON1 kit assay. We also performed method comparison for the research assay against glutathione peroxidase, a known antioxidant enzyme. These studies do not in any way cover all aspects of PON1 method validations; however, they will help to address some aspects of the enzyme performance. The outcome of the current study should encourage additional clinical studies to investigate other aspects of factors known to affect PON1 enzyme function and performance. A completed PON1 enzyme evaluation will provide conclusive evidence for its future utilization as biomarker in health and disease.

**Material and Methods**

Samples from an ongoing project were used for this validation study, approved by the Institutional Review Board (IRB) of the Medical Center of Central Georgia-Mercer University School of Medicine. Blood samples were collected from infertile women (mean age ±SD): 33.7±4.7 years old, range 26–44 years). Subject inclusion criteria in this study included presence of ovaries, regular menstrual cycle, nonsmoker, no history of immune system-related disorders, cardiovascular disease, diabetes, cancer, or a venous thrombo-embolic disorder. We excluded subjects, who either had recent acute systemic infection or were regularly using antihypertensive, antithrombotic, or lipid-lowering drugs.

**Material**

BD Vacutainer® tubes containing trisodium citrate, lithium heparin (Li Hep), sodium heparin (Na Hep), EDTA, clot activator gel, sodium fluoride (NaF), or plain tubes were purchased from VWR, Radnor, PA. All other chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

**Samples collection**

Blood samples were processed immediately; frozen samples were shipped in dry ice to the Department of Clinical Laboratory and Nutritional Sciences, College of Health Sciences, at the University of Massachusetts Lowell for subsequent analysis. Glutathione peroxidase and the commercial arylesterase/paraoxonase assays were performed according to the manufacturer’s instructions (ZeptoMetrix Corporation, NY). The research PON1 assay was performed as described previously by us and others [5,9,10]. In brief: Plasma PON1 arylesterase activity was measured using 1 mM pNPA (nitrophenyl acetate) as substrate, as assessed from the formation of p-nitrophenol at 410 nm at 37°C. Typically, aliquots of 10 μl of plasma were placed in microtiter plate wells in triplicate; the reaction was initiated by the addition of the substrate pNPA, to yield a
final concentration of 1 mM in PBS buffer containing calcium and magnesium. After mixing, the plate was read immediately to establish 0 time values, and the reactions were incubated at 37°C. Readings were recorded at the end of 30 min. PON activity was calculated using the Arylesterase/Paraoxonase Standard: (300 μl/kit); containing purified PON from the commercial kit [9].

**Linearity**

To determine the linearity of the research PON1 method, serial dilutions from plasma pool with PON1 range around 250 U/L was performed (6 dilutions). The resulting PON1 levels were plotted against the corresponding theoretical concentration. The linearity plot was visually evaluated for any indications of a nonlinear relationship between concentration and response.

**Precision studies**

Evaluation of precision performance for the research PON1 quantitative measurement method was performed as previously reported [12–14]. The repeatability of the closeness of agreement between results of successive measurements of PON1 was carried out under the same condition. Aliquots (40) of 5 pooled samples were used for the assessment of the repeatability and within-lab precision. Two aliquots were analyzed over a 20-day period.

**Effect of storage temperature studies**

To examine the effect of short-term storage on sample stability, we stored 3 aliquots of 20 samples at 4°C, −20°C, and −80°C for 3 months, and later samples were analyzed in triplicate for the determination of PON1 levels and activities using the lab-based research PON1 assay.

**Effect of type of anticoagulant**

To assess the effect of the use of anticoagulant on PON1 assay performance, we collected blood samples from 10 apparently healthy individuals for each sample type tube containing 1 of the following: BD Vacutainer® tube trisodium citrate, lithium heparin (Li Hep), sodium heparin (Na Hep), EDTA, sodium fluoride (NaF), clot activator gel, or plain tube. Blood was collected according to each BD Vacutainer® tube instruction. Plasma or serum was collected by centrifugation at 3000 rpm for 10 min. The research PON1 method was used to analyze the PON1 in samples in triplicate immediately after collection.

**Interferences**

Sample abnormalities due to the presence of hemolysis, icterus, and lipemia interfere in laboratory tests that use optical methods have been shown to affect analyte performance. We therefore measured PON1 in 10 samples spiked with hemolyzed or lipemic samples. Hyperlipidemic plasma sample 0.2 mL with Tg >500 mg was added to equal volume of serum sample, mixed and left to stand for 5 min. The mixture was analyzed and data were calculated after adjusting the dilution factor and PON1 levels in the samples. Similarly, 10 plasma samples where spiked with hemolysate of its red blood cells. The hemolysate was prepared as follows. Collected blood was centrifuged for 10 min at 3000 rpm. The plasma was removed and used for PON1 assay before spiking it with hemolysate. Remaining packed red blood cells (RBC) were washed 3 times with PBS to remove the buffy coat and any other plasma residues. Hemolysis was performed by pipetting out 0.5 ml of washed red blood suspension in ice-cold distilled water and later centrifuged at 3000 rpm to remove any RBC traces for another 10 min. Hemolysate was further diluted with PBS to make a 500 mg/dL hemoglobin in samples, as previously described [15]. Specimens were later assayed for the PON1 concentrations.

**Methods comparisons studies**

Method comparison studies were used for assessing the accuracy of the PON1 research method as compared to a commercially available kit method. This was performed as previously described [16]. Forty samples were analyzed simultaneously for both methods. We also compared the research method to a commercially available GSH-Px method using the same 40 samples.

**Statistical analysis**

Data analysis was performed using analysis protocol for each specific method mentioned above. Statistical analysis and plotting were performed using Analyse-it® for Microsoft Excel software. Pearson’s correlation analysis was used to evaluate the relationship between variables. Statistical differences of P≤0.05 were considered significant.

**Results**

Precision studies: the reproducibility of the PON1 enzymatic assay used in research labs was assessed (Figure 1). The 20-day PON1 assays precision for 2 sample replicates (mean ±SD) was 339.24±25.38, producing a 7.7% CV. The assay linearity at the 9.3–223.8 U/L range has a coefficient of R²=0.8821 (Figure 2). The Spearman correlation for the relationship between the storage at the various temperature conditions have shown a coefficient of R²=0.67 for the samples stored at 4°C, −20°C, and R²=0.56 for the 4°C vs. −20°C and R²=0.30 for the −20°C vs. −80°C (Figure 3A–3C). The effect of the type of anticoagulant
was studied in detail (Figure 4). Samples collected in fluoride and citrate anticoagulants had greater PON1 levels than the other anticoagulants. Samples in NaF, citrate, EDTA, clot activator, and sodium heparin had increased PON1 levels of 49%, 24.5%, 19.8%, 11.4%, and 8%, respectively, compared to the serum. Samples in lithium heparin had 10.4% decreases in PON1 levels compared to the serum. We studied the effect of some biological interference on PON1 performance such as sample hemolysis and higher lipids levels; these studies showed that hemolysis resulted in small increases in PON1 levels. The changes were incremental with the increases in the intensity of hemolysis, but these effects were not significant (Figure 5A). Samples spiked with lipids had 13% reduction in PON1 levels (Figure 5B). The method comparison studies performed between the commonly available method for PON1 assay and a similar non-ELISA commercially existing PON1 method resulted in a poor correlation performance, demonstrating a weak Spearman correlation producing a coefficient of $R^2=0.40$ for the range 104.9–245.6 U/L (Figure 6A, 6B). We also compared the plasma PON1 levels to a commercially available glutathione peroxidase method; the Spearman correlation coefficient
Effect of sample types on PON1 assay

Figure 4. The effect of the types of anticoagulants was showed that samples in NaF, citrate, EDTA, clot activator, and sodium heparin have increased PON1 levels of 49%, 24.5%, 19.8%, 11.4% and 8%, respectively, compared to serum. Samples in lithium heparin have 10.4% decrease in PON1 levels compared to serum.

Effect of hemolysis on PON1 assay

Figure 5. The hemolysis had small increases in PON1 levels, shown with the increases of the intensity of hemolysis (A). However, samples spiked with lipids showed 13% reduction in PON1 levels (B).

of $R^2 = -0.127$ indicates a poor correlation between the 2 antioxidant enzymes.

Discussion

PON1 enzyme activity is essential for the physiological function of several key metabolic pathways, and its plasma level is associated with the pathogenesis of many diseases. These findings have been reported in numerous clinical research studies [1,3,10]. Despite the outcomes of these clinical investigations showing PON1 as a promising biomarker, its plasma levels in clinical settings has not been utilized for clinical diagnostic or therapeutic management. The non-utilization of the PON1 assay in clinical management can be attributed to the lack of sufficient diagnostic evidence, which in part is linked to inadequate validation studies on its assay performance. Biomarkers validation studies are central components of the verification of any method performance. The use of a method in clinical applications requires sufficient information on the method precision and effect of sample type, which may be affected by the type of anticoagulants used. Also, since most of the biomarkers in clinical studies are assayed in batches, samples are usually stored for various time periods. Storage can affect sample matrix and therefore its quality, and assay values can thus be compromised. The current study was designed to address some of the factors associated with PON1 performance. Method accuracy and precision are regarded as the primary indicators of method effectiveness. Determination of these parameters helps to identify the extent of systematic and random errors associated with repeated determination of samples. Limited PON1 assay validation studies have been reported earlier [14,15]. We studied the assay linearity and precision in the commonly used PON1 assay. Our data indicate the PON1 arylesterase activity as measured from the formation of $p$-nitrophenol has intra- and inter-assay CVs of 2.1% and 7.7%, respectively, for a 20-day assay variability as measured using parallel aliquots of the same sample and within-assay
variability as measured from parallel aliquots every day the assay was performed. These data are comparable to a recently reported PON1 assay validation in large animals [15]. The assay has shown acceptable linearity (r=0.93). Since the current measurements were performed manually, improved linearity and precision data are achievable by the use of automation. Sample stability studies have indicated that PON1 is quite stable at 4°C, −20°C, and −80°C for 3 months. The small effect may result from the extended period of storage, as can be concluded from 4°C storage. This has also been reported earlier [17]. The PON1 assay storage for 2 years was shown to be stable if stored at −80°C, but according to this study the enzyme activity is known to decline after 2 years. Analyte stability in biological samples can be impacted by the sample matrix and possibly by the sample container; plastic containers are most commonly used for plasma storage and it is not clear if the use of glass tubes for sample storage will have different effects on the PON1 assay performance. To the best of our knowledge, the effect of anticoagulant type on PON1 assay performance has not been studied. To verify and compare the PON1 assay in different samples collected in various anticoagulants, we performed multiple assays of the same sample collected in containers with several different types of anticoagulant. Our results indicate that PON1 assay is affected by the type of anticoagulants. Samples on fluoride, citrate, and EDTA had a substantial increase in PON1 levels, while samples collected in clot activator or sodium heparin tubes had minimal increases compared to serum. Lithium heparin is the only anticoagulant that has shown a decrease in PON1 level compared to serum. This suggests that, in addition to collecting serum for PON1 assay, sodium heparin and the use of clot activator tubes may be used as well. However, if the PON1 level is desired for clinical determination, an adjustment of its levels between these tubes may be desirable. The anticoagulant studies can also be used for the determination of the enzyme activation or inhibitory agents. Interference experiments demonstrated that sample hemolysis has limited effect on the enzyme activity. This limited effect can be attributed to the optical affects; the ELISA-based assays may not be affected by the hemolysis. The presence of high lipid concentrations in plasma samples resulted in decreasing PON1 levels. This finding suggests that PON1 assay can be affected by hyperlipidemic samples; such samples may need to be diluted before being assayed for their PON1 levels. Lipemic samples are high in chylomicrons and VLDL particles, which are capable of masking light, resulting in false decreased PON1 levels. Lipemia is expected to interfere in PON1 assay, which uses the absorbance of light as part of the detection; however, a smaller effect of lipids is expected in the immunologically-based PON1 assay techniques. We compared the PON1 assay commonly performed in research settings, which usually requires manual reagent preparations, to a commercial PON1 kit that uses a similar method. Linear regression calculations used in method comparison studies have demonstrated that the 95% CIs for the slopes and intercepts for the 2 methods are statistically different. Given that the purpose of the method comparison studies is to assess inaccuracy, the current study showed systematic error at various levels between the 2 methods. More concerning is that the 2 methods are colorimetric-based assays. It is not clear how the lab research method will perform.
against the ELISA/EIA (enzyme immunoassays). Results of the method comparison between the commercial kit and the lab-prepared method do not reflect or criticize the effectiveness of the commercial kit, and additional investigation may be required to allow better assessment of the performance of the methods. We also compared the plasma PON1 assay to the GSH-Px assay. This study was primarily conducted to assess the relationship between these 2 antioxidant enzymes. Our analysis indicated no correlation between PON1 and GSH-Px in this set of samples. PON1 correlation with GSH-Px was not adequately studied. Recently published data have shown conflicting reports, while Krzystek-Korpacka et al. [18] reported that PON1 arylesterase activity decreased in general and central obesity, high blood pressure, and hyperinsulinemia conditions, and was correlated with superoxide dismutase, catalase, and glutathione peroxidase. Desai et al. [19] demonstrated that livers of non-alcoholic steatohepatitis (NASH) patients exhibited elevated mRNA expression of catalase and PON1, but not GPX1 or GSR. Data from our lab demonstrated that ovarian stimulation causes significant increase in serum PON1 and SOD, GPX activities in women undergoing IVF or IUI, and that the enhanced effects were positively correlated with E2 peaks. This shows that PON1 and GSH-Px independently increase in disease and may not essentially correlate in these listed diseases.

Conclusions

The current evaluation studies provide evidence of the factors that may contribute to PON1 performance and illustrate the impact of interference and the anticoagulant types on the assay. Additional studies, especially on the effect of drugs interference and the use of automation, are need. More comparative studies are essential, particularly to assess the emerging ELISA/EIA PON1 assays.

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