methods

Development of an immunoblot assay with infrared fluorescence to quantify paraoxonase 1 in serum and plasma

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Abstract Paraoxonase 1 (PON1) requires calcium for activity and is inactivated in the presence of EDTA. Because of this, studies to date have used serum or heparinized plasma for both activity and mass assays of PON1. Whole serum and EDTA plasma were analyzed by SDS-electrophoresis and Western blot using anti-PON1 monoclonal antibody 4C10. Because PON1 has one disulfide and one free cysteine residue, the samples were reduced with dithiothreitol before electrophoresis. Western blot identified a major PON1 band with a molecular mass of ~45 kDa and two minor bands of ~40 and 35 kDa in both serum and EDTA plasma. This established that PON1 is inactive, but structurally intact, in EDTA plasma and suggested that a mass assay could be developed based on SDS-electrophoresis and Western blot. Linearity was established for plasma and for a PON1 standard. Quantification was based on the major PON1 band at 45 kDa. The correlation between serum and plasma PON1 mass was 0.9553. The between-run variation was determined with a serum pool to be 7.8%. The mass of PON1 in serum was significantly correlated with arylesterase activity ($r = 0.85$). Thus, we have demonstrated the feasibility of measuring PON1 mass in either serum or EDTA plasma.—Connelly, P. W., G. F. Maguire, C. M. Picardo, J. F. Teiber, and D. Draganov. **Development of an immunoblot assay with infrared fluorescence to quantify paraoxonase 1 in serum and plasma.** ***J. Lipid Res.*** **2008.** 49: **245–250.**

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Human paraoxonase 1 (PON1) is a calcium-dependent serum enzyme that is found in association with HDL (1). PON1 has a variety of substrates and has been found to have multiple polymorphisms in both the coding sequence (L55M, Q192R) and in the noncoding sequence (2). Promoter polymorphisms, notably −108C/T (also referred to as −107C/T) and −162A/G, have been shown to affect PON1 expression (3–5).

Substrates, such as paraoxon, which are hydrolyzed at different rates by PON1 Q192 versus PON1 R192, have been used to phenotype PON1 and to determine possible sensitivity to organophosphate pesticides (3). However, these substrates are not of use for determining the mass of PON1 in serum. Arylesterase activity in human serum, measured using phenyl acetate hydrolysis, is expressed similarly by the known PON1 polymorphic forms and has been used as a surrogate for PON1 mass (1).

The first reported analysis of PON1 mass in normal subjects was by Blatter Garin et al. (6), who compared subjects from Geneva and Manchester and identified significant differences in PON1 Q192R allele frequency, PON1 enzyme activity, and PON1 mass. Studies of PON1 knockout and transgenic mice, and of human populations, are consistent with PON1 having a protective function in organophosphate toxicity and cardiovascular disease (7, 8). Although PON1 is thought to be tightly associated with apolipoprotein A-I (apoA-I), the concentration and activity of PON1 have been consistently reported to vary over a wider range than those observed for apoA-I. This variation may be the result, in part, of environmental factors. For example, smoking (inversely) (9) and moderate alcohol intake (positively) (10) have been reported as determinants of PON1.

Investigation of PON1 using EDTA plasma has been actively discouraged because of the loss of enzyme activity in the presence of EDTA as a result of the removal of calcium from the enzyme. Because a number of studies have only EDTA plasma samples available, we considered

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that it would be of value to develop an assay for PON1 mass to allow application to a broader range of investigations. We report here the validation of an assay for PON1 mass in EDTA samples.

MATERIALS AND METHODS

SDS-electrophoresis

Polyacrylamide gels (8% acrylamide, 29:1 ratio of acrylamide to bis-acrylamide) containing 12 wells, prepared in Novex cassettes, were used for the separation of components of serum and EDTA plasma. An 80-fold dilution of serum or plasma was made with 60 mM Tris, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, and 50 mM dithiothreitol and heated at 95°C for 5 min. A 10 μl volume of sample was loaded into each well (equivalent to 125 nl of serum). Recombinant human PON1 (rHuPON1) with an N-terminal hexa-histidine tag was purchased from Cedarlane Laboratories (Hornby, Ontario, Canada) and was used as the primary standard. The gels were electrophoresed at 150 V for 20 min and at 200 V for 40 min using a Novex Mini gel cassette. Prestained molecular mass markers (Fermentas Pageruler prestained protein ladder No. SM1811) were used to identify the molecular mass region between 35 and 55 kDa. The gel was cut between these markers, and the gel zones from up to four gels were placed on a nitrocellulose membrane for electrotransfer using a Novex Mini apparatus. Transfer was performed at 30 V for 90 min. The membranes were blocked in 10 ml of Li-Cor Odyssey (Li-Cor, Omaha, NE) blocking buffer diluted 1:1 with Tris-buffered saline (blocking buffer) and then incubated in blocking buffer containing 0.1% Tween 20 and anti-human PON1 monoclonal antibody 4C10 [antibody 606.4C10.28 is available from J. F. Teiber (john.teiber@utsouthwestern.edu) or from the University of Michigan Hybridoma Core Facility (hybridoma@umich.edu)] diluted 1:10,000 from a stock solution containing 9.9 mg antibody/ml. The membrane was incubated with gentle rotational mixing overnight at room temperature and then washed four times with 5 min incubations in Tris-buffered saline and 0.1% Tween 20. The second antibody, Alexa Fluor 680 anti-mouse IgG (Alexa catalog No. A21065), was diluted 1:10,000 with blocking buffer, 0.1% Tween 20, and 0.01% SDS. The membrane was incubated with 10 ml of this solution with gentle rotational mixing at room temperature for 1 h. The membrane was then washed six times for 5 min each time with Tris-buffered saline and 0.1% Tween 20 and then twice for 2 min each time with Tris-buffered saline. The membrane was then scanned with the Li-Cor Odyssey.

Arylesterase assay

Sample (3–5 μl) was added to 20 mM Tris, pH 8, 1 mM $CaCl_2$ and 10 mM phenyl acetate (final volume, 1 ml) in a quartz cuvette, rapidly mixed by repeated inversion, and read at room temperature at 270 nm with a Spectronic 1001 Plus spectrophotometer (Milton Roy) every 3 s after a 3 s delay, for a total of 30 s.

Human serum and plasma samples

Fasting blood samples were obtained with informed consent according to a protocol approved by the St. Michael's Hospital Research Ethics Board. Samples obtained from volunteers, who were part of a larger study of familial apoA-I deficiency, were used for this study. Serum and EDTA plasma samples were divided into aliquots and stored at −70°C before analysis and thawed a single time for analysis of arylesterase activity and PON1 mass. Lipids, lipoproteins, and apolipoproteins were assayed on the samples at the time of sample collection. ApoA-I and apoB were reassayed on the thawed aliquot, and the values were in agreement with those obtained at the time of sample collection. Anonymized serum samples obtained from subjects attending the St. Michael's Lipid Clinic were used to assess the potential interference from lipemia. ApoA-I and apoB were assayed using the Dade-Behring BN100 Plasma Protein analyzer. Cholesterol and triglyceride were assayed using a Hitachi 917 random-access analyzer and Roche reagents. HDL cholesterol was assayed in the supernatant after dextran sulfate-Mg^{2+} precipitation. All lipid assays were standardized by the National Heart, Lung, and Blood Institute-Centers for Disease Control Lipid Standardization Program (Atlanta, GA).

Statistical analysis

Statistical analysis was performed with GraphPad Prism version 5.0 (GraphPad, San Diego, CA).

RESULTS

Western blot analysis, using anti-human mouse PON1 monoclonal antibody 4C10, for a serum-plasma pair is shown in **Fig. 1**. The advantage of using the prestained gel markers can be seen, as they are detectable by infrared fluorescence. The pattern for PON1 with a major band of ~45 kDa and one minor band of 40 kDa was identical between serum and plasma and identical to the pattern reported previously by Kuo and La Du (11), who reported that the 40 kDa form of PON1 was indistinguishable from endoglycosidase H-treated 45 kDa PON1. Two

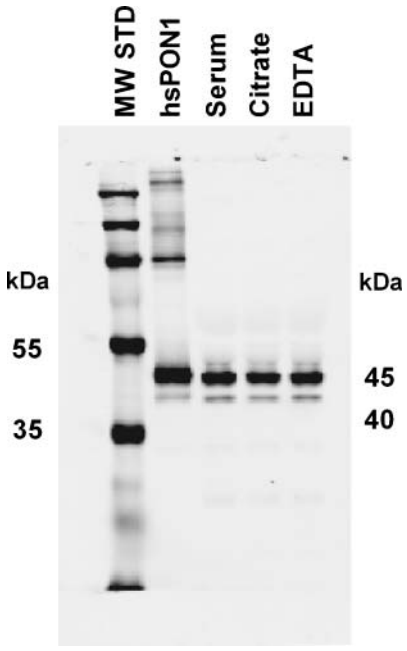

Fig. 1. SDS-electrophoresis and Western blot for paraoxonase 1 (PON1) in serum, citrated plasma, and EDTA plasma. MW STD, molecular mass standard; hsPON1, PON1 Q192 purified from human serum; Serum, human serum collected without anticoagulant; Citrate, human plasma collected in the presence of citrate anticoagulant; EDTA, human plasma collected in the presence of EDTA anticoagulant.

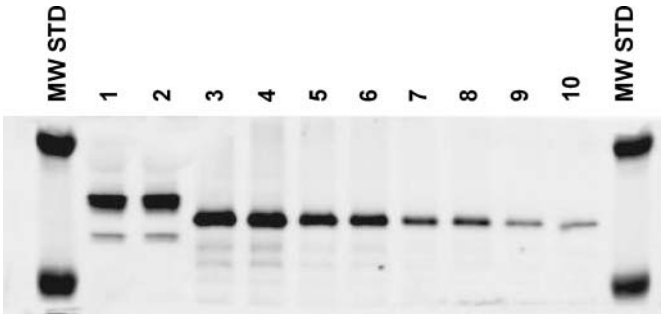

Fig. 2. SDS-electrophoresis and Western blot for the PON1 standard curve obtained with *E. coli*-derived recombinant PON1. The portion of the SDS gel between the 55 and 35 kDa molecular mass markers (MW STD) was transferred to a nitrocellulose membrane and Western blotted as described in Materials and Methods. Lanes 1 and 2, duplicates of the serum calibrator. Lanes 3 through 10, PON1 standard in concentrations as follows: lanes 3 and 4, 50 ng of PON1; lanes 5 and 6, 25 ng of PON1; lanes 7 and 8, 12.5 ng of PON1; and lanes 9 and 10, 6.25 ng of PON1.

other primary antibodies were tested and found to be less sensitive and thus not satisfactory for a quantitative assay of PON1.

The presence of albumin caused distortion of the PON1 band when the equivalent of 1 μl of plasma was electrophoresed. A plasma sample volume of 125 nl was optimal for eliminating the effect of albumin on the electrophoresis while providing sufficient sample for the detection of PON1.

PON1 192Q purified from human serum, baculovirus-expressed PON1 192Q, and *Escherichia coli*-expressed PON1 192Q were tested as calibrators. Each gave linear responses (r^2 = 0.987; 6.25–50 ng of *E. coli*-expressed PON1) (**Fig. 2**). The *E. coli*-expressed PON1 was chosen as a calibrator because it is available commercially and thus should allow replication and transferability of the method between laboratories. The mass value assigned by the manufacturer was used for each set of Western blots and was used to assign a target value to the reference plasma.

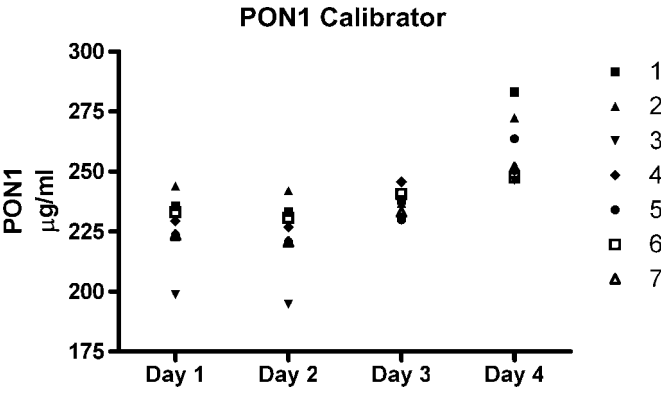

Fig. 3. Plots showing results for a reference serum run on 4 separate days (labeled as Day 1–Day 4) and within day by gel (identified as 1–7). The reference serum was run in duplicate, and average values are plotted. A value of 236 μg/ml was assigned to this serum calibrator.

TABLE 1. Study subject characteristics

Variable	Mean	Minimum	Maximum
Age, 13 males/7 females (years)	39.5	20	67
Cholesterol (mmol/l)	5.62	2.86	9.0
Triglyceride (mmol/l)	1.60	0.46	4.49
VLDL cholesterol (mmol/l)	0.78	0.05	3.29
LDL cholesterol (mmol/l)	3.62	1.69	5.8
HDL cholesterol (mmol/l)	1.22	0.73	2.12
Apolipoprotein A-I (g/l)	1.50	1.07	2.03
Apolipoprotein B (g/l)	1.36	0.59	2.19
Arylesterase (U/l)	193	103.8	280.8
Paraoxonase 1, serum (μg/ml)	171	90.9	292.1

We evaluated between-day variation as follows. Each day samples were loaded on four gels, with one gel containing the molecular mass marker in the outside lanes and plasma calibrator samples in duplicate and an eight-point standard curve. The remaining three gels contained the molecular mass marker in the outside lanes, the plasma calibrator sample in duplicate, and each unknown (study subject) sample in duplicate. Thus, 15 unknown samples were assayed per four gels. One technician can routinely run eight gels per day. The results for the plasma calibrator run on 4 separate days and on seven gels per day are shown in **Fig. 3**. By two-way ANOVA, between-day variance accounted for 58% of the variation ($P < 0.0001$) and

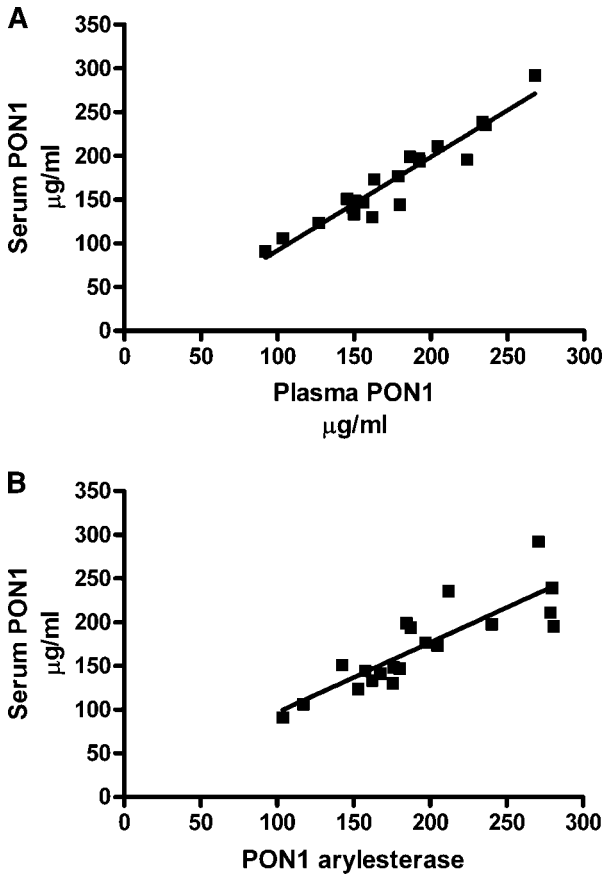

Fig. 4. A: Correlation of PON1 mass in serum-plasma pairs. B: Correlation of serum PON1 mass with serum arylesterase activity.

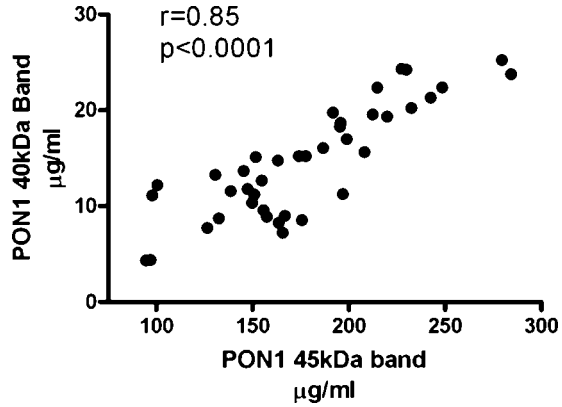

Fig. 5. Scatterplot of the concentration of the 45 kDa PON1 band versus the 40 kDa PON1 band. The data points are individual determinations for serum and plasma from 20 samples.

between-gel variance accounted for 28% of the variation ($P = 0.0017$) in the reference plasma sample. The within-day coefficient of variance ranged from 1.9% to 6.6%, and the coefficient of variance for all analyses was 7.8%. The mean value of 236 µg/ml was assigned to the reference plasma and used as the calibrator for each gel.

The characteristics of the subjects are summarized in **Table 1**. The results for 20 serum-plasma pairs are shown in **Fig. 4**. The correlation between serum and plasma was 0.95, and there was no statistically significant difference between serum and plasma values (paired *t*-test $P = 0.36$). The correlation between serum arylesterase activity and PON1 concentration was 0.85 for serum and 0.88 for plasma ($P < 0.0001$) (Fig. 4).

We examined the relationship between PON1 mass, PON1 activity, and lipoprotein concentrations. Serum PON1 mass explained 72% of the variation in PON1 arylesterase activity. Plasma PON1 mass explained 77% of the variation in PON1 arylesterase activity. Of the lipid and lipoprotein variables (Table 1), only HDL cholesterol (8%) or apoA-I (7.2%) contributed significant additional information ($P < 0.03$) with regard to the variation in arylesterase activity.

The proportion of the minor PON1 band was relatively constant over the range of PON1 concentrations, with a correlation of 0.85 ($P < 0.0001$) (**Fig. 5**). However, the intensity of the minor band was near the value of the lowest standard and therefore should be considered an approximate value.

The interference by high concentrations of triglycerides was tested because there may be an upper limit to the solubilization of triglycerides by SDS. The protocol followed for the results shown in Fig. 5A–C involves mixing sera in the precise ratios of 1:0, 1:3, 1:1, 3:1, and 0:1. The individual results of duplicate determinations are shown. A linear relationship indicates the absence of interference. High concentrations of triglyceride (>10 mM) affected the linearity of the assay (**Fig. 6A**) ($P = 0.048$ for

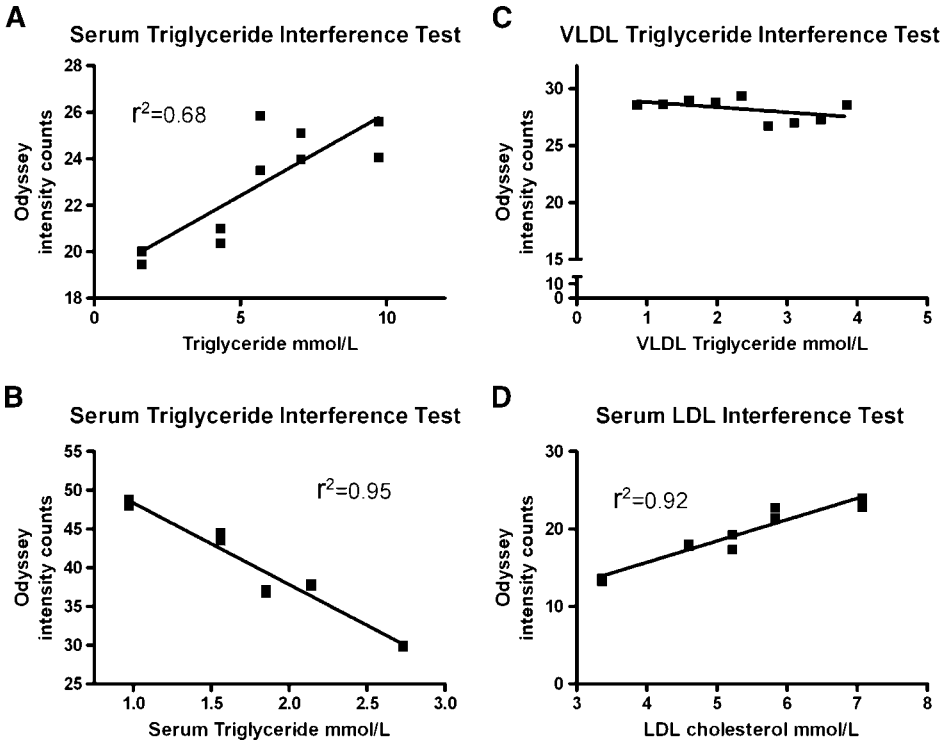

Fig. 6. Results for tests of the linearity of PON1 mass determined in the presence of varying concentrations of triglycerides (A, B), VLDL (C), or LDL cholesterol (D). Linearity was tested using human specimens according to the Clinical and Laboratory Standards Institute, formerly National Committee for Clinical Laboratory Standards, document EP6-P ("Evaluation of the Linearity of Quantitative Analytical Methods"). A description of this protocol can be found at http://www.westgard.com/lesson26.htm (accessed August 1, 2007).

deviation from linearity). Because this concentration of triglyceride interfered with the assay, it is not possible to determine whether the higher value of PON1 in the serum with a triglyceride of 10 mM was a true value. The assay was shown to be linear for triglyceride concentrations up to 3 mmol/l by mixing of whole serum samples (Fig. 6B). This was confirmed by increasing the triglyceride concentration to 4 mM by the addition of VLDL to a sample with a low concentration of triglyceride (Fig. 6C) ($P = 0.19$ for slope not different from 0). LDL cholesterol concentrations up to 7 mM did not affect the assay (Fig. 6D).

DISCUSSION

The method described here for the quantification of PON1 has several important features: *1*) the method is sensitive, using only nanoliter quantities of serum or plasma; *2*) the use of infrared fluorescence for the detection and quantification provides a signal that is stable over time and linear, allowing the scanning of the blots to be carried out at a convenient time; and *3*) the method allows the use of a single primary antibody, either monoclonal or polyclonal.

The use of an ELISA for the quantification of PON1 mass was first reported in 1994 (6). The method was calibrated using purified PON1 and Western blot. The current method improves and extends the original method as outlined above. Because of the limited accessibility of mass assays for PON1 (12), many studies restrict the analysis of PON1 to activity measurements. It has been suggested that measurement of PON1 should routinely include the concentration of the enzyme (13). The relative importance of enzymatic activity, as assessed by hydrolysis of a selected substrate under saturating conditions, enzyme mass, and enzyme specific activity, remains to be determined. Some studies have found differences in all, some, or only one of these characteristics (6, 9, 14–25). Recently, it was reported that PON1 mass inversely predicts mortality in patients on hemodialysis (26). Thus, the indications for the measurement of the mass of PON1 continue to expand. Measurement of PON1 will facilitate its use as a biomarker to predict risk for vascular disease.

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