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Original article

Development of an automated method for the determination of human paraoxonase1 activity

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Background: Human plasma paraoxonase1 (PON1) is an esterase catalyzing the hydrolysis of organophosphorus pesticides and other xenobiotics. The aims of this study were to develop a rapid method to determinate PON1 activity, evaluate some interference, and study the influence of storage temperature on PON1 activity assay.
Methods: Measurement of PON1 activity was performed for 369 samples by measuring the hydrolysis of paraoxon using a spectrophotometric method adapted on konelab 30□.
Results: The developed method facilitates the determination of PON1 activity at the rate of more than 200 samples per hour, and it is linear between 2 and 900 IU/L. Intra and inter-assay imprecision coefficients of variation were 2% and 5% respectively. PON1 activity in serum was correlated with those in heparinized plasma ($r = 0.994$, $p < 0.001$) and in plasma/EDTA ($r = 0.962$, $p < 0.001$). The mean inhibition of the PON1 activity was, by EDTA/K_3, 41 ± 10 %. There was not significant PON1 activity variation after 40 days of storage at -20°C or at +4□C. There were no substantial interferences from haemoglobin, jaundice and hyperlipidemia.
Conclusion: The developed method is reliable, reproducible, and suitable. It can also be performed on heparinized plasma for the determination of PON1 activity. Hence, it may be useful for assaying PON1 activity in several intoxications such as organophosphorus, sarin, and soman nerve agents.

Keywords: Automated method, paraoxonase1 activity, organophosphate pesticides and nerve agents intoxication

Human plasma paraoxonase1 (PON1; aryldialkylphosphatase; EC 3.1.8.1) is a calcium-dependent esterase associated with high-density lipoprotein (HDL). This enzyme received its name from paraoxon, parathion's toxic metabolite, which is one of its most studied substrates. This enzyme hydrolyzes the active metabolites of several other organophosphorus (OP) insecticides, nerve agents such as sarin and soman, as well as oxidized lipids and pharmaceutical drugs [1].

PON1 catalytic mechanism and physiological functions are still unclear. The level of human PON1 in circulation has been reported to correlate with resistance to organophosphates, suggesting that PON1 acts as a bioscanvenger [2]. Recently, PON1 is regarded as a promising catalytic scavenger for the pre-treatment and therapy of OP poisoning including warfare nerve agents and pesticides [3]. In addition, PON1 is involved in drug metabolism and drug inactivation [1].

PON's association with HDL in serum led to the suggestion that the enzyme might have a role in lipid metabolism and protect against the development of atherosclerosis [4]. PON1 antioxidant activity is well established and low PON1 activity has been reported in oxidative stress and in the aetiology of various disorders, including cancers [5], cardiovascular diseases [6, 7], Parkinsonism [8, 9], Alzheimer disease [10, 11], diabetes [12,13] and renal failure [14, 15].

Thus, the measurement of serum PON1 activity has become a viable biomarker of PON1 phenotypic variation in the epidemiological studies. Additionally, it helps to minimize the exposure of susceptible individuals to organophosphorus compounds [1, 16].

Many previous studies have established the experimental condition for PON1 substrate-specific assays by determining the appropriate substrates

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[7, 17, 18]. However, several other factors can affect biomarker variability such as the biological factors and the technical variations such as variation in specimen handling and storage. The aims of this study were to develop a rapid automated method to determinate PON1 activity, evaluate the interferences from triglycerides, heamolysis, and jaundice on PON1 activity and to study the influence of storage temperature and the number of freeze-thaw cycles, which may be an additional source of technical variation, in PON1 activity.

Material and methods

Blood collection

Blood samples (n = 369) were collected from 123 healthy volunteers via venipuncture and were drawn, into tubes without anticoagulants, into tubes with ethylendiamine-tetraacetic acid potassium salt (EDTA/K_3), and into tubes with lithium heparin. Serum or plasma was aliquoted after centrifugation and then stored at -20°C until analysis.

Three pools with low, intermediate, and high PON1 activities were stored at -20°C to study the influence of freeze-thaw cycles on PON1 activity. Other pools of plasma were also prepared to study conservation temperature effects on PON1 activity. The aliquots were conserved in different temperatures (ambient temperature, +4°C and -20°C).

Two other pools with low and high PON1 activities were prepared to study the interferences from triglycerides, haemolysis, and jaundice on PON1 activity, by supplementing each pool with triglycerides, haemoglobin, or bilirubin at various concentrations, ranging from 0.4 to 11.8 mmol/L, 0.27 to 4.25 g/L and 25 to 500 µmol/L, for each parameter respectively.

Determination of PON1 activity

The measurement of PON1 activity was performed in both serum and plasma samples by measuring the hydrolysis of paraoxon using a spectrophotometric method adapted on konelab 30□ automate (Thermo-Clinical Lab System). Serum or plasma (5µL) was added to freshly prepared tris-NaOH buffer (0.26 M, pH 8.5) containing 0.5 M NaCl, 1.2 mM paraoxon and 25 mM calcium chloride. After thirty seconds of incubation at 37°C, the liberation of p-nitrophenol ($\varepsilon = 18.053\ M^{-1}cm^{-1}$) was followed at 405 nm for six minutes (at 54 second intervals). One unit of activity is defined as the amount of enzyme producing 1 µmol of p-nitrophenol per minute. All samples were analysed in triplicate and the mean of the three obtained values was used for subsequent analyses.

The inter- and intra-assay imprecision were examined by analysing multiple aliquots of heparinized plasma from volunteer samples. All the samples were assayed in triplicate. Intra-assay was examined with the replicate analysis performed in the same run. For the inter-assay imprecision, the aliquots of the same sample stored at -20 °C were analyzed on different days. Variation coefficients (CV) were then determined to evaluate assays imprecision.

Statistical analysis

PON1 activities were log-transformed to normalize their distributions. The Pearson's correlation coefficient was used to compare PON1 enzyme activity measured in serum and heparinized plasma or the one with EDTA. The student "t-test" was used to evaluate the differences between PON1 activities in serum and those in plasma. CVs were calculated to evaluate inter and intra-assay variability and examine the effects of multiple freeze-thaw cycles on enzyme activity.

Results

Performances of PON1 activity assay

The developed method facilitates the determination of PON1 activity at the rate of more than 200 samples per hour.

It is linear up to 900 IU/L, and the regression line of expected versus observed values was Y = 0.9989 X + 0.3151 and r = 0.9998.

Intra-assay imprecision was determined with replicate analysis of pools performed in the same run, and the CV of measurements was about 2%. To assess the inter-assay imprecision, the samples were analysed on consecutive days and the CV was equal to 5%.

The average intra-assay calculated from measurements of each sample was approximately 2% for both sample types, and the average CV between paired serum and plasma samples, for the same subject, was 3%.

The effect of specimen type on PON1 activity

PON1 activity was measured in 123 samples, and it was determined for each sample in serum, plasma collected on EDTA/K_3 (plasma/EDTA) and heparinized plasma (**Table 1**). Although PON1 activity

in heparinized plasma was lower than that in serum, there were no significant differences between PON1 activities on serum and heparinized plasma (p = 0.8). However, a significant decrease was found between PON1 mean activities in plasma/EDTA when compared to activity in serum (p = 0.001).

A high correlation was found between enzyme activity in serum and that in heparinized plasma (r = 0.994, p <0.001). PON1 activities in serum were also correlated with plasma/EDTA. The Pearson's correlation coefficient was 0.962 and 0.959 for serum versus plasma/EDTA and heparinized plasma versus plasma/EDTA respectively (p <0.001 for all assays) as shown in **Figure 1**.

Figure 2 illustrates the inhibition enzyme activity (%) in plasma/EDTA samples vs. serum samples of the same individuals. The inhibition of the PON1 activity, by EDTA/K_3, was between 30 and 50 % as compared to enzyme activity in the serum samples with a mean inhibition equal to 41 ± 10 %.

Effects of conservation temperatures on PON1 activity

The aliquots of the same heparinized plasma pools were used to examine the effects of conservation temperatures (ambient, + 4°C, -20°C) on PON1 activity. The activity was assayed in these aliquots for 40 days. **Figure 3** illustrates PON1 mean activity at the three different temperatures. No significant decrease was found in the mean activity at the three aliquots for 15 days.

Starting from day 17, a significant decrease was noted for PON1 activity in plasma stored at ambient temperature (p <0.001). However, no significant variations were noted for PON1 activity, after 40 days when the samples were stored at -20°C or at +4°C.

The effect of freeze-thaw cycles on PON1 activity

Three pools were used to examine the effects of freeze-thaw cycles on PON1 activity in specimens stored at -20°C. The aliquots of each pool underwent 10 freeze-thaw cycles. No significant variation was found after these freeze-thaw cycles (**Figure 4**). The CV determined to examine the effect of multiple freeze-thaw cycles ranged from 1 to 13%.

Interferences

Interferences from haemolysis, jaundice and triglycerides were assessed at two pools with low and high PON1 activities (**Table 2**). There were no substantial interferences from haemoglobin (≤4 g/L), jaundice (bilirubin ≤500 µmol/L), and hyperlipidemia (≤12 mmol/L).

Discussion

PON1 activity was measured by a simple and rapid automated method adapted on Konelab 30□. Low intra and inter-assay CVs were found for the studied method (lower than six percent). According to Marsillash et al. [19], the variation coefficients of the measurement ranged from 11.2 to 17.7%.

There were no significant differences between PON1 activities in serum and heparinized plasma. Besides, a high correlation was found in PON1 activities between these two types of specimens. Although lithium is an inhibitor of PON1 activity [6], this inhibition is negligible and PON1 activity can be measured in lithium-heparin treated samples. Our results were consistent with those reported by Ferr□ et al. [20], who also indicate that heparinized plasma samples may be used for the study of PON1 because the effect of lithium-heparin on PON1 measurement is relatively small. However, Huen et al. [21] reported that PON1 enzyme activities were significantly higher in serum than in heparinized plasma. Activities measured in plasma are often lower than those obtained in serum because fibrin retains some water and the serum becomes more concentrated [6].

It is well established that in presence of EDTA, PON1 (calcium-dependent esterase) loses its ability to reactivate due to the loss of its stability [6]. However, it is important to know whether this inhibition

Table 1. Mean PON1 activities in different specimen types

Specimen type	Mean activity (IU/L)	p[a]
Serum	185 ± 66	-
Heparinized plasma	181 ± 65	0.8
Plasma/EDTA	115 ± 69	0.001

[a] PON1 activities compared to those of corresponding serum samples

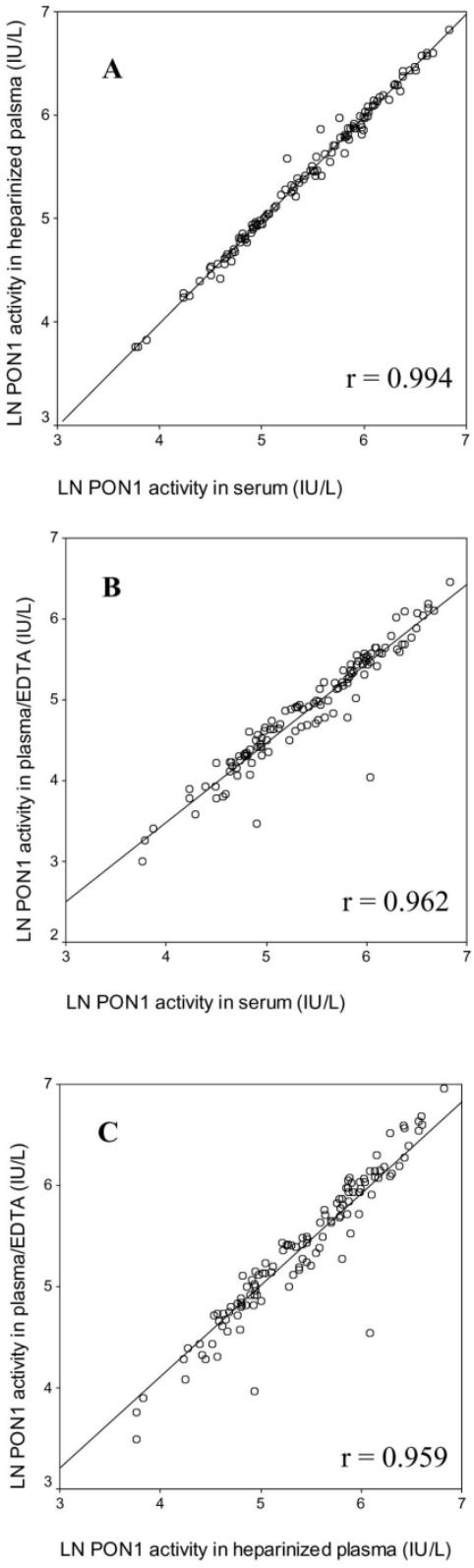

Figure 1. PON1 mean activities in serum vs. heparinized plasma **(A)** vs. plasma/EDTA **(B)** and in heparinized plasma vs. plasma/EDTA **(C)** $p < 0.001$ for all assays. r is the Pearson's correlation coefficient.

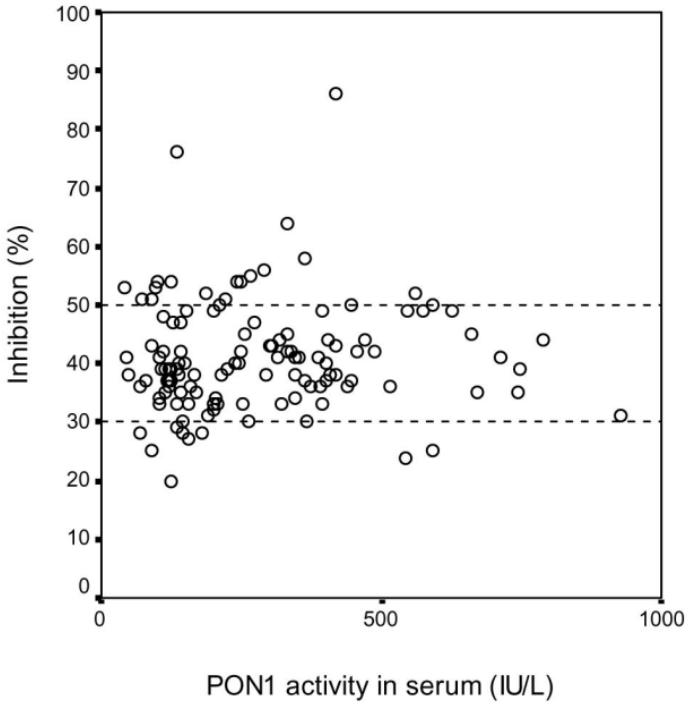

Figure 2. Inhibition PON1enzyme activity (%), by EDTA/K_3, in plasma/EDTA samples vs. serum samples of the same individuals.

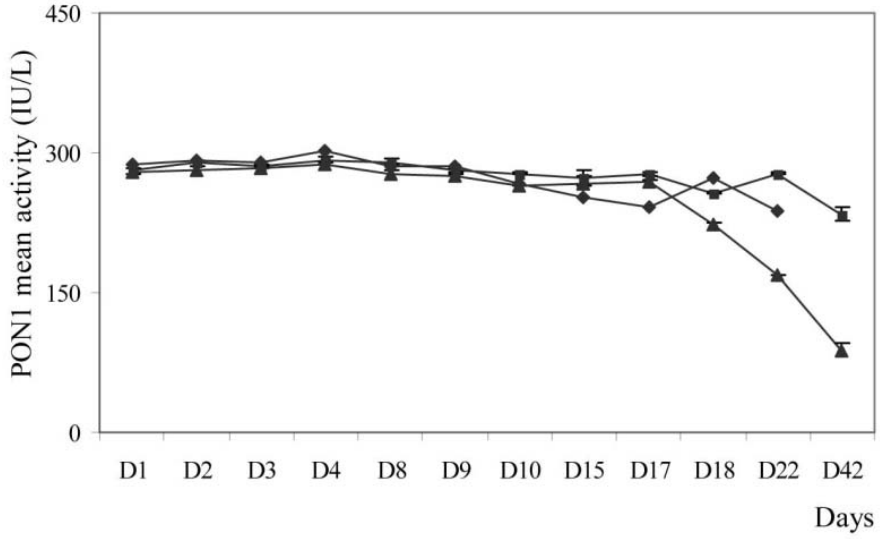

Figure 3. Mean PON1 activity at three different temperatures: ambient temperature (▲), +4°C (■) and -20□C (◆).

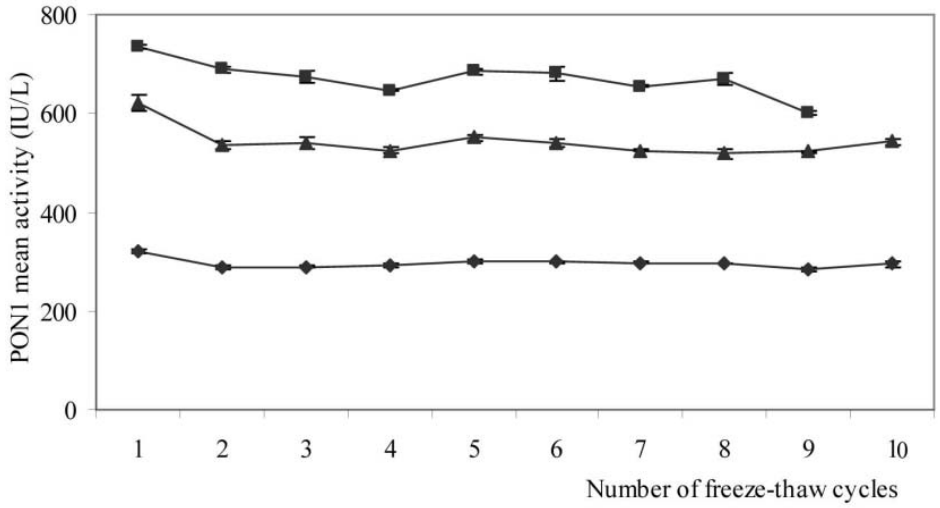

Figure 4. Effect of freeze-thaw cycles on PON1 activity, in three pools with low activity, pool 1 (◆); intermediate activity, pool 2 (▲) and high activity, pool 3 (■).

Table 2. Percentage recovery, for study of interferences of haemoglobin, jaundice, and hyperlipidemia on PON1 activity (%).

Hemoglobin (g/L)	0.27	0.53	1.06	2.12	3.18	4.25
Pool 1	99	98	102	96	95	96
Pool 2	98	99	100	101	100	103
Mean (SD)	99 (1)	99 (1)	101 (1)	99 (4)	98 (4)	100 (5)
Bilirubin (mmol/L)	25	50	100	250	375	500
Pool 1	94	89	90	92	93	94
Pool 2	99	99	99	101	100	103
Mean (SD)	97 (4)	94 (7)	95 (6)	97 (6)	97 (5)	99 (6)
Triglycerides (mmol/L)	0.4	1.5	3.0	5.9	11.8	-
Pool 1	100	101	97	95	97	-
Pool 2	100	102	99	100	101	-
Mean (SD)	100 (0)	102 (1)	98 (1)	98 (4)	99 (3)	-

is total or not in natural conditions. In our study, the mean inhibition of PON1 activity, by EDTA, was 41%. Mackness [6] reported that PON1 activity determined in 12 apparently healthy subjects was much lower in plasma collected in tubes containing EDTA when compared to activity in corresponding serum samples, representing an 88% inhibition. According to Charlton-Meny et al. [22], the addition of 0.02 mmoL/L EDTA to eight serum samples before addition of paraoxon substrate, containing 2 mmoL/L calcium, led to a 60% decrease in PON1 activity. However, the addition of EDTA to a final concentration, which is higher than that already mentioned above, did not inhibit the PON1 activity. Furthermore, we found that PON1 activities in plasma collected in EDTA tubes, stored at +4°C for only one night, were almost nil (data not shown). This finding indicates that the inhibition of PON1 activity by EDTA is time-dependent. Hence, PON1 activity can be, if necessary, determined in plasma samples collected in EDTA tubes in the same day of blood collection, or stored immediately at -20°C until analysis, taking into account the inhibition percentage.

According to Mackness [6], the reactivation of an enzyme inhibited by EDTA can only be performed by adding an amount of excess of calcium, under certain conditions, and that the enzyme could not be reactivated by the removal of EDTA, because calcium remains bound to the EDTA.

In our study, PON1 enzyme activity was stable in plasma samples stored at ambient temperature for 15

days. Over this period, a significant decrease was noted in enzyme activity. A negligible variation was noted in PON1 activity, after 40 days, when the samples were stored at -20 °C as well as at +4 °C. In addition, no significant variations were found after 10 freeze-thaw cycles on PON1 enzyme activity. These findings indicate that PON1 activity is a stable enzyme that resists temperature fluctuations. Our results corroborated with those reported by Huen et al. [21] who also indicated that multiple freeze-thaw cycles (up to four) did not have any effect on PON1 enzyme activity.

Some recent studies investigated the activity, function and thermal stability of human PON1 (23, 24). PON1 activities and stability depend decisively on the enzyme molecular environment. Besides, Rochu et al. [24] discovered that the human phosphate binding protein (HPBP) is a major actor in maintaining the functional state, storage and thermal stability of purified human PON1.

In the present study, lipidemia, jaundice, and haemolysis appeared to have no influence on the assay, and the measurement of PON1 activity is reliable in patients with several diseases. This finding is in agreement with the results reported by Marsillach et al. [19], who studied the influence of these interferences on enzyme activity in patients with liver impairment.

Conclusion

In this study, the developed fast-automated method is reliable, reproducible, and suitable for measuring PON1 activity. Furthermore, no substantial interferences by serum states were found. There were no significant differences between PON1 activities in serum and heparinized plasma. Therefore, the latter is an acceptable alternative for the study of PON1 activity. However, EDTA/plasma could not be used to investigate PON1 activity, but if necessary, PON1 activity must be measured immediately after blood collection and mean inhibition percentage must be taken into account in interpreting the obtained values.

Although PON1 is a stable enzyme that resists temperature fluctuations, freezing is the most appropriate mode for conservation especially multiple freeze-thaw cycles without any significant changes. The developed method is useful for assaying PON1 activity in a large number of patients with several diseases, especially as PON1 has been implicated in many important afflictions affecting health, including organophosphorus sensitivity, and in the etiology of various disorders such as diabetes, atherosclerosis, Parkinson's, and Alzheimer diseases.

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The authors have no conflict of interest or commercial interests to declare.

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