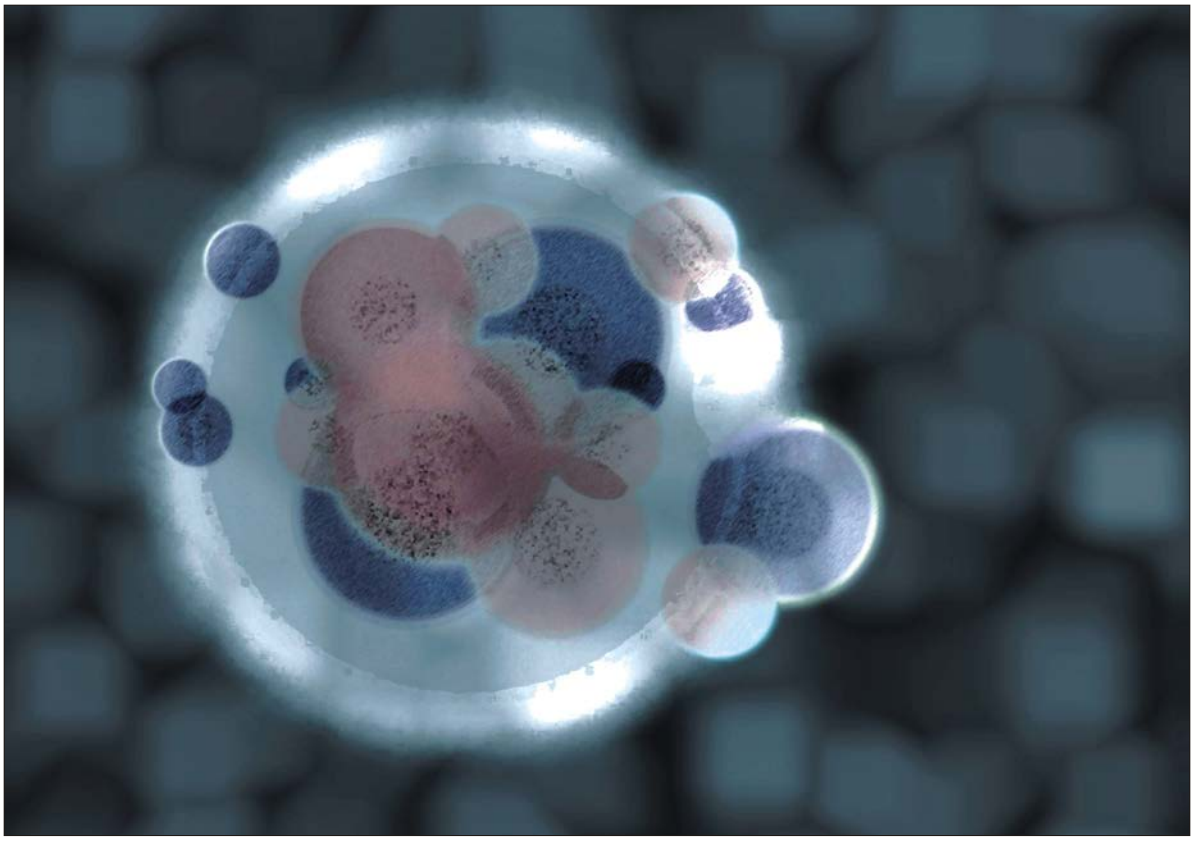

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Evaluation of Polymorphism at Codon 192 of Paraoxonase 1 on its Kinetic Behavior

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Abstract: Human paraoxonase 1 (PON1), a High-Density Lipoprotein (HDL)-associated esterase has been implicated in slowing down the development of atherosclerosis. In the present study, kinetic and inhibition studies on PON1 were conducted to assess three parameters: the Michaelis constant (K_M) and maximal rate of metabolisme (V_{max}) of paraoxonase and inhibition constant (K_i) of phenylacetate. Human paraoxonase 1 (PON1) activity was measured spectrophotometrically at 405 nm, using plasma samples in basal (without added NaCl) and salt-stimulated assays with 1 M NaCl. Inhibition studies were performed using phenylacetate as an inhibitor of PON1 in basal assays, pH 8.0. Estimates of K_M and V_{max} were obtained from the Lineweaver-Burk plot. Estimates of K_i were obtained from the secondary plot of apparent K_M ($K_{M,app}$) versus inhibitor concentration. The parameter values were evaluated for the genotypes $PON1_{192QQ, -QR, -RR}$. In salt-stimulated assays, the V_{max} increased two-fold for the $PON1_{192QQ}$ samples and three to five-fold for the $PON1_{192RR}$ samples compared with basal assays. Similarly, it increased four-fold for $PON1_{192QR}$ samples. Michaelis constant (K_M) was comparable with or without 1.0 M NaCl across the three genotypes. The Lineweaver-Burk and Dixon plots revealed that phenylacetate was a predominantly competitive inhibitor exhibiting linear mixed type inhibition. The K_i values were also comparable across the three genotypes. We conclude that the three kinetic parameters of PON1 in the Malaysian population estimated in the present report were comparable with those reported by other studies on PON1 from Caucasian populations.

Key words: Enzymatic activity, inhibition constant, maximal rate, Michaelis constant

INTRODUCTION

Human paraoxonase 1 (PON1, EC 3.1.8.1) catalyzes the hydrolysis of paraoxon to the nontoxic products, p-nitrophenol and diethylphosphoric acid (Costa *et al*., 2005). PON1 has been implicated as a predictor of coronary heart disease (Roest and Voorbij, 2008). Human paraoxonase 1 (PON1) activity towards paraoxon (POase), is distributed trimodally (with respect to the polymorphism at codon 192) (Jarvik *et al*., 2003). In Caucasian populations, the frequency of the $PON1_{192R}$ allele is lower (Hofer *et al*., 2006) compared to Asian populations such as the Malay, Chinese and Korean populations (Poh and Muniandy, 2007; Zhang *et al*., 2006).

The estimation of enzymatic characteristics of PON1 has been done by kinetic studies. Kinetic and inhibition studies on PON1 have been reported in a number of studies using initial reaction rates to assess three common parameters: the Michaelis constant (K_M), maximal rate of metabolism (V_{max}) of PON1 and inhibition constant (K_i) of phenylacetate as an inhibitor of paraoxon (Du *et al*., 2001; Eckerson *et al*., 1983b). These parameters were usually evaluated based on the PON1 genotypes in previous studies and differences in the three parameters with respect to the genotypes have been reported by Geldmacher-von Mallinckrodt *et al*. (1979) and Gan *et al*. (1991). In addition, when sodium chloride was added to the kinetic assay, the V_{max} of PON1 was consistently higher compared to basal assay conditions without sodium chloride (Smolen *et al*., 1991). The Q and R isozymes showed differences in K_M values for paraoxon (Smolen *et al*., 1991). Phenylacetate was shown to act as an inhibitor of PON1 activity towards paraoxon and the inhibition was of the mixed type (Eckerson *et al*., 1983b) although kinetic studies did not involve secondary plots.

In inhibition studies for a one-substrate reaction, it is assumed that catalysis occurs via., the formation of a complex between enzyme, E and substrate, S. This complex breaks down to give product, P and regenerate E. The scheme below assumes that the inhibitor-containing complexes were in equilibrium with each other. K_{ES}, K_{ESI}

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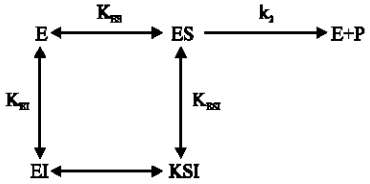
and K_{EI} represent dissociation constants of ES, ESI and EI, respectively, while k_2 represents the conversion of ES to E and P.



In its reciprocal form, a general kinetic equation was derived as shown in Eq. 1.

$$\frac{1}{v}=\left[\frac{1}{V_{max}}\right]\left[1+\frac{[I]}{K_{ESI}}\right]+\left[\frac{K_{ES}}{K_{EST}}\right]\left[1+\frac{[I]}{K_{EI}}\right]\left[\frac{1}{[S]}\right] \quad (1)$$

In competitive inhibition, assuming that $K_{ESI} = 8$ (the ES complex cannot combine with inhibitor, I nor the EI complex with S), then Eq. 1 is reduced to:

$$\frac{1}{v}=\left[\frac{1}{V_{max}}\right]+\left[\frac{K_{ES}}{V_{max}}\right]\left[1+\frac{[I]}{K_{EI}}\right]\left[\frac{1}{[S]}\right] \quad (2)$$

The intercept at the abscissa of this type of plot would then be represented by $-1/K_M\ (1+[I]/K_{EI})$. If inhibition is of the mixed type with predominant competitive traits, the intercept would be:

$$\left[\frac{1+\frac{[I]K_M}{K_{EI}K_M'}}{K_M\left[1+\frac{[I]}{K_{EI}}\right]}\right]$$

where, K_M' is the Michaelis constant modified in presence of excess inhibitor, as shown in Eq. 3 below:

$$\frac{1}{v}=\left[\frac{1}{V_{max}}\right]+\left[\frac{K_{ES}}{V_{max}}\right]\left[\frac{1+\frac{[I]K_M}{K_{EI}K_M'}}{K_M\left[1+\frac{[I]}{K_{EI}}\right]}\right]\left[\frac{1}{[S]}\right] \quad (3)$$

In this study, the kinetics of human serum PON1 was investigated by measuring initial rates of hydrolysis to observe the effects of PON1 polymorphisms on the kinetic behavior of PON1.

MATERIALS AND METHODS

Kinetic and inhibition studies on PON1 were conducted in the current investigation to assess three parameters: the Michaelis constant (K_M) and maximal velocity (V_{max}) of PON1 and inhibition constant (K_i) of phenylacetate. The velocity of PON1 was determined by measuring the apparent rates of reaction spectrophotometrically (Copeland, 2000). Samples displaying high POase activity within the highest tertile were selected for the $PON1_{192RR}$ group, whereas those with low POase activities well within the lowest tertile were selected for the $PON1_{192QQ}$ group as suggested by Mueller *et al.* (1983) in order to clearly define the kinetic values based on genotype.

PON1 catalyses the following hydrolysis reaction:

$$\text{Paraoxon} \xrightarrow[Ca^{2+}]{PON1} \text{p} - \text{Nitrophenol} + \text{diethylphosphoric acid}$$

The rate of hydrolysis of paraoxon was monitored by measuring the liberation of the product p-nitrophenyl phosphate at 405 nm.

Spontaneous hydrolysis: A blank determination of basal assay mixture (containing Tris.Cl, pH 8.5 and $CaCl_2$) without plasma was first performed to observe spontaneous hydrolysis of paraoxon. Plastic rectangular cuvettes of 1.0 cm light path were used. All measurements were conducted at room temperature (21.2±1°C). Paraoxon-ethyl-PESTANAL or paraoxon (Sigma Chemical Co., St. Louis, MO) was added to 1.0 mL basal assay buffer (without added NaCl), pH 8.5, in a 1.5 mL microfuge tube to a final concentration of 1.0 mM. Paraoxon was prepared fresh every hour. One milliliter of stock 1.0 mM paraoxon solution was placed in a plastic cuvette containing the assay buffer. The cuvette was covered with a 0.5 square piece of parafilm and inverted 5 times to mix. The cuvette was immediately placed in a UV-Vis spectrophotometer (Varian Cary 50, Varian Inc. Scientific, California). The measurement of activity was performed by using the Kinetics application of the Cary WinUV Version 3.00 software.

Estimation of K_M and V_{max}: Frozen stored plasma samples were thawed, mixed and centrifuged at 13,000 x g for 1 min to remove particulate matter prior to use in kinetic studies. The substrate dependence of PON1 velocity was determined from the initial rates of hydrolysis run at different paraoxon concentrations. The experiments were run both in the absence and presence of 1 M NaCl, pH 8.5. Substrate: Paraoxon was added to 1.0 mL assay buffer in a 1.5 mL microfuge tube to a final 1.0 mM. The tube was capped securely and shaken vigorously. As before, paraoxon was prepared fresh and used within 1 h.

- **Sample:** The plasma sample was diluted 1:10 in dilution buffer

- **Procedure:** Stock 0.01 to 0.2 mL of 1.0 mM paraoxon solution was first placed in a plastic cuvette containing 0.89 to 0.70 mL of the assay buffer

The diluted plasma was then introduced to the paraoxon-assay buffer solution, mixed and measured spectrophotometrically. The initial rate of hydrolysis was measured at 405 nm at room temperature, with readings continuously taken for 2 min. The initial linear rates were used in calculations for POase activity. The accuracy of the computerized calculations was evaluated by the method of Richter *et al.* (2004). One unit of enzyme activity is expressed as 1 μmol of product/min and reported as U/L. PON1 velocity at different substrate concentrations were used to generate the double reciprocal Lineweaver-Burk plot of 1/v versus 1/[S], where [S] is the concentration of paraoxon in mM and v is the velocity in U/L. Estimates of K_M and V_{max} were obtained directly from the plot. As paraoxon is a potent cholinesterase inhibitor, all paraoxon-containing waste was hydrolyzed in 10 M NaOH solution overnight and washed down with copious amounts of water (Mueller *et al.*, 1983).

Inhibition studies: Phenylacetate was used in inhibition studies of PON1. As NaCl inhibits arylesterase activity, a blank determination without both salt and plasma was performed to observe the spontaneous hydrolysis of the diluted phenylacetate solution (Senti *et al.*, 2001). Inhibition studies were then performed using phenylacetate (Sigma Chemical Co., St. Louis) as an inhibitor of PON1. To prepare the inhibitor stock solution to a final concentration of 10 mM, phenylacetate was dissolved in basal assay buffer, pH 8.0 by continuous stirring for 2 h. Stock solutions of phenylacetate (1, 0.5, 0.3 and 0.2 mM) were then prepared. Stock phenylacetate inhibitor solution was placed in a plastic cuvette containing the assay buffer. Paraoxon solution (1.0 mM) was then added. Finally, the diluted plasma was introduced to the assay mixture, mixed and monitored spectrophotometrically. PON1 activity with paraoxon as the substrate in the presence of phenylacetate as an inhibitor was calculated. The extinction coefficient at pH 8.0 was 17/mM/cm as used in the calculations (Richter *et al.*, 2004). The calculated PON1 activity was used to generate the Lineweaver-Burk plot to yield apparent K_M ($K_{M,app}$) values at each inhibitor concentrations. The K_i value was determined from a secondary plot of $K_{M,app}$ versus inhibitor concentration. The mode of phenylacetate inhibition was determined by using the Dixon plot (Dixon and Webb, 1964) of 1/v versus inhibitor concentration.

Statistical analysis: Comparisons of three or more subgroups were performed by using either one-way Analysis of Variance (ANOVA) or the nonparametric Kruskal-Wallis test. Analysis of Variance (ANOVA) was used in the analysis of data when equal variance was assumed whereas Kruskal-Wallis test was used for data when unequal variance was assumed. In cases of significant difference between groups, the post hoc analysis was used to identify, which group(s) of samples differed significantly from the others. Tukey's post hoc test was performed if the variances were similar, whereas Dunnett's T3 post hoc test was used when variances were different. Statistical analyses in general were performed using Statistical Package for the Social Sciences (SPSS) 11.0 for Windows software (SPSS, 2002). A p-value of <0.05 was taken to be statistically significant.

RESULTS AND DISCUSSION

Spontaneous hydrolysis: Table 1 shows that rates of spontaneous hydrolysis for paraoxon and phenylacetate were negligible in a typical experiment.

Estimation of K_M and V_{max}: The increase in absorbance due to PON1 activity was linear for at least 2 min of the assay duration. The linearity allowed for the calculation of activity based on initial rates. POase activities for typical samples from the $PON1_{192QQ}$, $_{-QR}$ and $_{-RR}$ genotypes are shown in Fig. 1. The selected $PON1_{192RR}$ samples had high POase activity, whereas $PON1_{192QQ}$ samples had low POase activity. $PON1_{192QR}$ samples had intermediate activity.

The double reciprocal Lineweaver-Burk plots for estimation of K_M and V_{max} for $PON1_{192QQ}$, $PON1_{192QR}$ and $PON1_{192RR}$ samples are collectively shown in Fig. 2 for comparison.

Effect of 1.0 M NaCl, on K_M and V_{max}: The K_M values obtained from assays with or without 1.0 M NaCl were comparable as the differences were not significant (paired samples t test, $p = 0.510$). There was significant correlation between the basal and salt-stimulated K_M

Table 1: Spontaneous hydrolysis of phenylacetate and paraoxon

Type of substrate	Rate of spontaneous hydrolysis, Mean±SD (U L^{-1})	Rate of hydrolysis in presence of plasma (U L^{-1})	Difference* (%)
Phenylacetate	0.00205±0.00025	33	6.2×10^{-5}
Paraoxon	0.00033±0.00014	700	4.7×10^{-7}

*The percentage of difference is calculated as follows: (Rate of hydrolysis-rate of spontaneous hydrolysis)/(Rate of hydrolysis)×100%

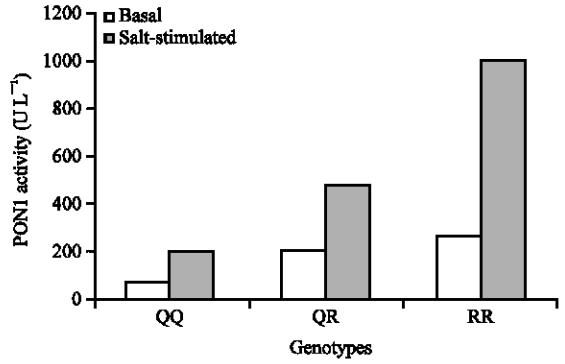


Fig. 1: Bar chart representing POase activity for typical samples from the $PON1_{192QQ, -QR, -RR}$ genotypes. The values were derived from plots of rate of absorbance with time. Note the progressively increasing activity from basal to salt-stimulated assays across these genotypes

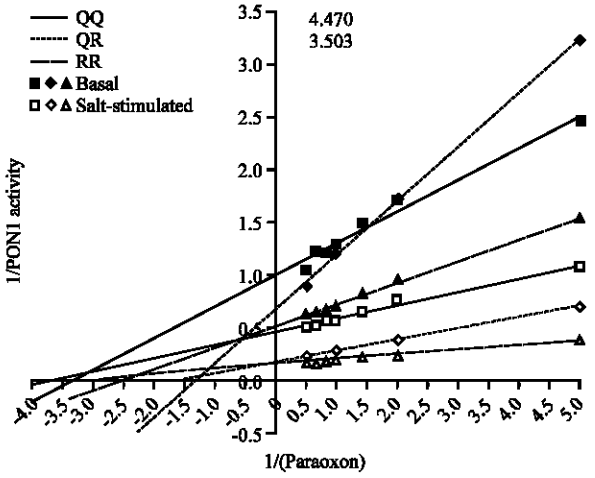


Fig. 2: Estimation of K_M and V_{max} in basal and salt-stimulated assays in three typical $PON1_{192QQ, -QR, -RR}$ samples. The y intercept corresponds to the reciprocal of V_{max}, whereas the x intercept corresponds to the reciprocal of K_M

(Pearson's correlation, p = 0.01). However, V_{max} values are significantly increased in the presence of NaCl (paired samples t-test, p = 0.002) by two- to fivefold. V_{max} was increased in salt-stimulated assays by three- to fivefold for the $PON1_{192RR}$ samples, approximately fourfold for $PON1_{192QR}$ samples and about twofold for the $PON1_{192QQ}$ samples compared to basal assays.

Effect of PON1 genotype on K_M and V_{max}: The K_M was compared in all three genotypes as shown in Table 2. The apparent discrepancies in K_M values between genotypes

Table 2: K_M values in comparison with three genotype

	K_M, mM (range)	
Genotypes	Basal*	1.0 M NaCl†
QQ	0.576±0.182	0.528±0.175
	(0.303-0.667)	(0.280-0.690)
QR	0.335±0.092	0.314±0.028
	(0.270-0.400)	(0.294-0.333)
RR	0.454±0.224	0.584±0.357
	(0.278-0.781)	(0.323-1.111)

Values are Mean±SD (range). One-way ANOVA, *p = 0.383, †p = 0.512

Table 3: V_{max} in comparison with three genotype

	V_{max}, U L^{-1} (range)	
Genotypes	Basal*	1.0 M NaCl†
QQ	121±25	253±29
	(102-159)	(222-286)
QR	194±3	742±39
	(192-196)	(714-769)
RR	294±102	1197±247
	(154-400)	(833-1370)

Values are Mean±SD (range). One-way ANOVA, *p = 0.028, †p<0.001

was not significant (one-way ANOVA, p = 0.383 and 0.512 for basal and salt-stimulated assays, respectively).

The V_{max} value was increased significantly according to the $PON1_{192QR}$ polymorphism in the following order (Table 3). QQ<QR<RR, in both basal and salt-stimulated assays. The salt-stimulated assay showed a higher degree of increment in activity compared to the basal assay.

Inhibition studies: In the Lineweaver-Burk plot, straight lines at different fixed inhibitor (phenylacetate) concentrations intersected at a common intersection point at the y intercept (Fig. 3a-c) for all three $PON1_{192QR}$ genotypes, a characteristic signature of a competitive inhibitor.

The Lineweaver-Burk plots were used to estimate K_M to build the secondary plot in the determination of K_i as shown simultaneously in Fig. 4 for all three genotypes.

The mean K_i values for $PON1_{192QQ, -QR, -RR}$ genotypes determined from secondary plots of $K_{M,app}$ versus phenylacetate concentration were as follows: 0.25±0.14 (0.13-0.44) mM, 0.27±0.22 (0.12-0.42) mM and 0.40±0.08 (0.31-0.49) mM, respectively (Fig. 5). The difference in K_i between genotypes was not significant (ANOVA, p = 0.308).

Dixon plot (1/v versus phenylacetate concentration) lines at different fixed paraoxon concentrations intersected in the second quadrant (Fig. 6a-c).

A replot of the Dixon plot slopes vs. phenylacetate concentration passed close to the origin for all three $PON1_{192QQ, -QR, -RR}$ genotypes (Fig. 7).

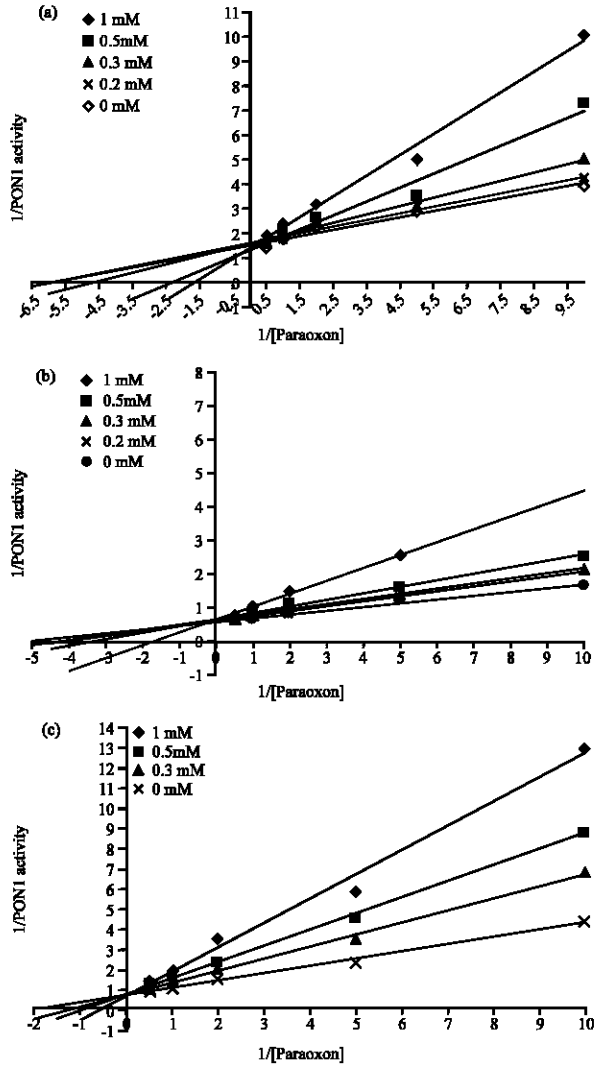


Fig. 3: Lineweaver-Burk plots at five concentrations of phenylacetate for a typical (a) $PON1_{192QQ}$ sample, (b) $PON1_{192QR}$ sample and (c) $PON1_{192RR}$ sample. The pattern of straight lines with intersecting y intercepts was characteristic of a competitive inhibitor

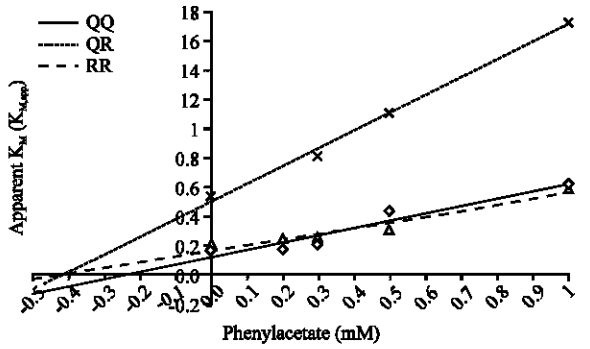


Fig. 4: Secondary plots of $K_{M,app.}$ as a function of inhibitor concentration for a competitive inhibitor (phenylacetate). The plots for typical $PON1_{192QQ}$, $_{QR}$, $_{RR}$ samples were shown here. The value of the inhibitor constant K_i was determined from the negative value of the x intercept of this type of plot

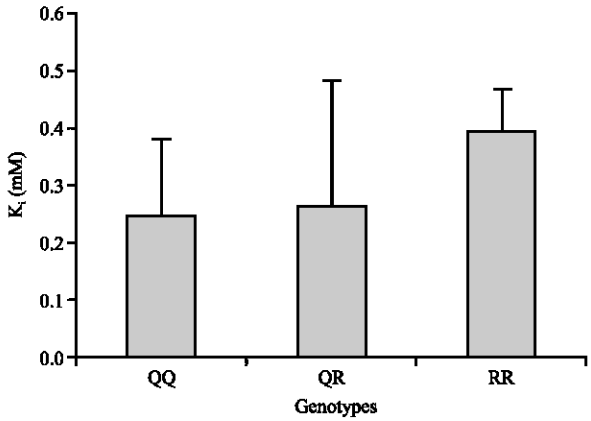


Fig. 5: K_i as a function of genotype. The inhibitor constant K_i was determined from the secondary plot of $K_{M,app.}$ as a function of inhibitor concentration. Bars and error bars represent mean of K_i and SD, respectively

Kinetic studies may be an approach that may be used to characterize PON1, based on quantitative measurements of the rate of hydrolysis of paraoxon (Dixon and Webb, 1964). Inferences may be made about the mechanism of PON1 action.

Spontaneous hydrolysis: Spontaneous hydrolysis of paraoxon and phenylacetate apparently were shown to be insignificant in this study. Thus, assays for PON1 activity were not routinely corrected for spontaneous hydrolysis. In this respect, it is relevant to note that Eckerson *et al.* (1983a) showed that spontaneous or nonenzymatic hydrolysis may be considered negligible except in samples having extremely low activity.

Estimation of K_M and V_{max} values: When the effect of NaCl on K_M was explored, it was seen that the apparent K_M value was comparable with or without 1.0 M NaCl for all three genotypes of $PON1_{192QR}$ polymorphism. Although K_M values for low PON1 activity samples (QQ) were on average slightly higher than for high (RR) or intermediate PON1 activity samples (QR), the apparent difference in K_M values between genotypes was not significant. In

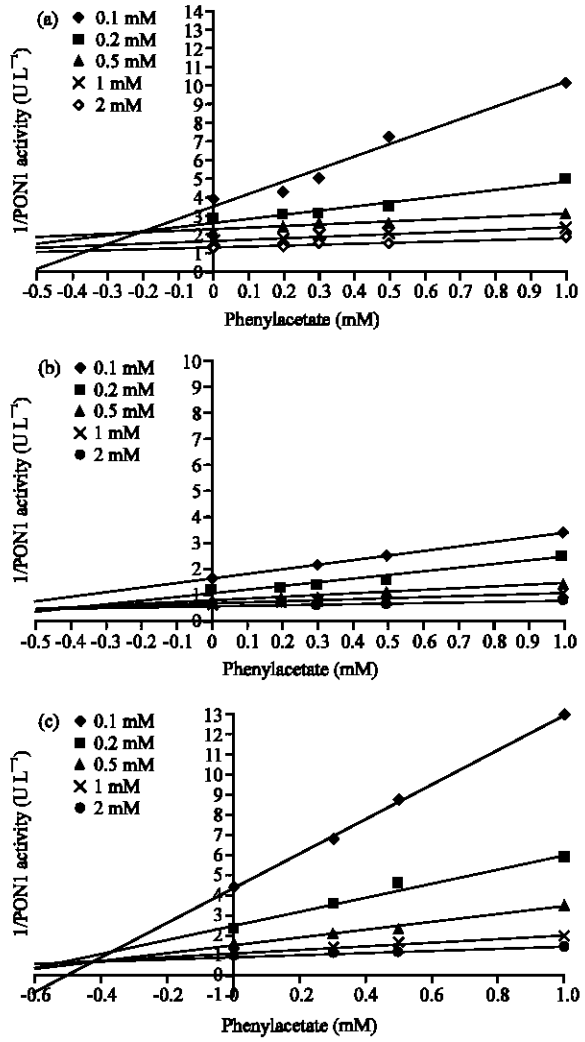


Fig. 6: Dixon plot for a typical (a) $PON1_{192QQ}$ sample, (b) $PON1_{192QR}$ sample and (c) $PON1_{192RR}$ sample

addition, the difference within the genotype was not significant either. This is in agreement with Mueller *et al.* (1983), who demonstrated that K_M was constant for the homozygotes for both low- and high-activity alleles. A slight increase in salt-stimulated K_M of $PON1_{192RR}$ samples compared to the basal counterpart was also observed. On the other hand, a slight decrease in K_M was observed in the $PON1_{192QQ}$ samples. The slightly higher K_M values of $PON1_{192QQ}$ samples compared with $PON1_{192RR}$ samples concurred with the study by Mueller *et al.* (1983). However, Eckerson *et al.* (1983a) showed that K_M values of $PON1_{192QQ}$ samples were lower compared to $PON1_{192RR}$ samples. Hence, various studies have reported minor differences in both genotypes with respect to K_M as shown in Table 4. Although, Smolen *et al.* (1991)

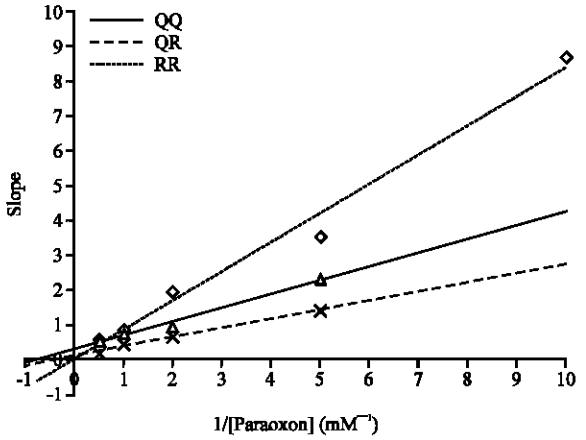


Fig. 7: Dixon slope replots for typical $PON1_{192QQ}$, -QR, -RR samples. The slopes of the Dixon plots were replotted as a function of 1/[paraoxon]. The replots passed close to the origin for all three genotypes indicative of mixed type inhibition

concluded that the Q and R alleles showed differences in K_M values for paraoxon, the observation was not supported by statistical analysis.

When the effect of NaCl on V_{max} was examined, it was shown that NaCl significantly increased V_{max} by two- to five-fold. This observation has been attributed to either an increased number of active sites or increased turnover number of the enzyme, but not to a change in the binding constant or concentration of paraoxon as the effect of salt has a rapid onset and is also rapidly reversible (Eckerson *et al.*, 1983a).

Inhibition studies: In purely competitive inhibition, the Lineweaver-Burk plots show straight lines at different fixed inhibitor concentrations intersecting at a common point at the y intercept. However, as the mode of inhibition of phenylacetate is of the mixed type, the apparent K_M was increased by a factor:

$$\left[\frac{1+\frac{[I]K_M}{K_{EI}K_M'}}{K_M\left[1+\frac{[I]}{K_{EI}}\right]}\right]$$

The inhibitor, in effect, causes the formation of the EI or PON1-phenylacetate complex. The slopes of the lines, given by $K_{M,app}/V_{max,app}$, vary among the lines because of the effect imposed on K_M by the inhibitor the degree of perturbation of K_M depending on the value of K_i.

Initial evaluation suggested that the type of inhibition was predominantly competitive. This was

Table 4: Comparison of K_M, V_{max} and K_i values

Authors	Samples	K_m (mM) (pH 8.5) Basal	K_m (mM) (pH 8.5) Salt	V_{max}, (U L^{-1}) (pH 8.5) Basal	V_{max}, (U L^{-1}) (pH 8.5) Salt	K_i, mM (pH 8.0)	Notes
Eckerson *et al.* (1983a, b)	High activity (B)	0.460	0.460	734	1670	0.77	PON assays: 412 nm, 25°C, 0.05 M glycine buffer, 1 mM paraoxon, pH 10.5
	Low activity (A)	0.430	0.430	-	-	0.55	Interaction studies: 10 mM Tris/HCl buffer, pH 8.0. Human serum.
Mueller *et al.* (1983)	High activity	0.420 (0.3-0.4)	-	420	-	-	PON assays: 405 nm, 37°C, 0.05 M glycine-Na glycinate buffer, pH 10.5.
	Low activity	0.460 (0.35-0.75)		-	200	-	Serum
Smolen *et al.* (1991)	High activity	0.271	0.21	659	-	-	PON assays: 412 nm, 25°C, 1 mM paraoxon, 50 mM glycine buffer, pH 10.5.
	Low activity	0.503	0.47	344	-	-	V_{max} in µmol/min/mg. Purified isozymes were used
Gan *et al.* (1991)	High activity	0.271	-	-	-	0.885	412 nm, 1 mM paraoxon, 50 mM Tris/HCl buffer, pH 7.4 and 8.0; 50 mM glycine/ NaOH, pH 10.5. Purified isozymes.
	Low activity	0.503	-	-	-	0.401	
Davies *et al.* (1996)	High activity	-	-	-	1769	-	Mean PON activity, not V_{max}
	Intermediate	-	-	-	977	-	
	Low activity	-	-	-	328	-	
Billecke *et al.* (2000)	High activity, R	-	-	741#	-	-	#Specific activity in µmol/min/mg
	Low activity, Q	-	-	870#	-	-	Purified PON1 was used
Zhu *et al.* (2006)	Serum-purified hPON1	0.54	-	-	-	-	405 nm, $E_{405} = 1805\ M^{-1}cm^{-1}$
	PON1 variants from *E. coli*	0.8	-	-	-	-	
This study	High, RR	0.45	0.58	294	1197	0.39	PON assays: 405 nm, 21°C, 10 mM Tris; Cl buffer, pH 8.5.
	Intermediate, QR	0.35	0.31	194	742	0.27	Interaction studies: 10 mM Tris/HCl buffer,
	Low, QQ	0.58	0.53	121	253	0.25	pH 8.0. Human plasma

shown by the extrapolating lines of different fixed paraoxon concentrations in the Dixon plot. These intersected in the second quadrant, thus excluding competitive or uncompetitive mechanisms and in agreement with the earlier studies by Eckerson *et al.* (1983b) and Gan *et al.* (1991).

Further analysis revealed that the replot of the Dixon plot slopes vs. the reciprocal of paraoxon concentration passed slightly over origin, confirming the previous observation that the inhibition was not purely competitive but of the linear mixed type. Gan and Eckerson's studies support the presence of a single enzyme in the human serum which accounts for both POase and arylesterase activities.

The K_i determined in the present study for phenylacetate was relatively low when compared to other studies as shown in Table 4. Gan *et al.* (1991) reported K_i in the range of 0.401-0.885 mM whereas Eckerson *et al.* (1983b) reported 0.55-0.77 mM. A trend that was apparent was that the K_i values decreased from high- to low-activity genotypes although the differences were not statistically significant.

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