Pharmacokinetics and Toxicodynamics of Pralidoxime Effects on Paraoxon-Induced Respiratory Toxicity

Pascal Houzé,† Donald E. Mager,‡ Patricia Risède,§ and Frédéric J. Baud†§

*Biochemistry Laboratory, AP-HP, Saint Louis Hospital, Assistance Publique-Hôpitaux de Paris, 75010 Paris, France; †INSERM U705, UMR 7157, Faculté de Pharmacie, Universités Paris 7 and 5, 75005 Paris, France; ‡Department of Pharmaceutical Sciences, University at Buffalo, SUNY, Buffalo, New York 14260; and §Department of Medical and Toxicological Critical Care, Lariboisière Hospital, University Paris Diderot, Assistance Publique-Hôpitaux de Paris, 75010 Paris, France

1To whom correspondence should be addressed at Biochemistry Laboratory, Hôpital Saint Louis, Assistance Publique-Hôpitaux de Paris, 1 Avenue Claude Vellefaux, 75010 Paris, France. Fax: +33 1 42 49 92 47. E-mail: pascal.houze@sls.aphp.fr.

Received February 26, 2010; accepted May 7, 2010

Empirical studies suggest that the antidotal effect of pralidoxime depends on plasma concentrations with therapeutic effects associated with concentrations above 4 mg/l. The purpose of this study was to determine the pharmacokinetic-toxicodynamic (PK-TD) relationships for the antidotal effect of pralidoxime on paraoxon-induced toxicity in rats. Diethylparaoxon inactivation of whole-blood cholinesterase activity was studied both in vitro and in male Sprague-Dawley rats. Toxic-induced respiratory effects were measured via whole-body plethysmography in control and pralidoxime-treated animals (50 mg/kg im injection). In the in vitro analysis, cholinesterase reactivation by pralidoxime in blood-poisoned diethylparaoxon (10nM) was proportional to the logarithm of drug concentrations. A mechanism-based TD model was developed, which well described the inhibition of cholinesterases by diethylparaoxon and reactivation with pralidoxime. The in vitro pralidoxime EC50 was estimated to be 4.67 mg/l. Animals exposed to diethylparaoxon exhibited a decrease in respiratory rate and an increase in expiratory time, and pralidoxime treatment resulted in a rapid complete but transient (<30 min) correction in respiratory toxicity. In contrast, there was a fast and total reactivation of blood cholinesterase activity over the 210-min study period. The in vivo TD model was extended to capture the time-course of in vivo pralidoxime antidotal effects, which explained the complex relationship between drug exposure and pharmacological response profile. This study provides insights into the role of oxime-rescue of paraoxon-induced toxicity, and the final PK-TD model might prove useful in optimizing the design and development of such therapy.

Key Words: diethylparaoxon; pralidoxime; plethymography; rat; pharmacokinetic; toxycodynamic.

Organophosphates (OPs) are used daily throughout the world as pesticides. They remain a major health concern reflected in the large number of annual acute poisonings (Eddleston et al., 2008; Satoh, 2006). According to the World Health Organization, there are annually more than 3 million OP intoxications, resulting in more than 200,000 deaths (Eddleston, Gunnell, et al., 2005, 2008; Jeyaratnam, 1990). Furthermore, chemical terrorism, involving organophosphates as nerve gas agents, has become a major concern. Indeed, sarin was used in two attempts of terrorist attacks in Japan in the nineties (Morita et al., 1995; Suzuki et al., 1995), and there have been allegations of the use of nerve agents such as tabun and sarin during the Iraq-Iran war in the mideighties (Balali-Mood and Shariat, 1998; MArs, 2007).

The early and intermediate phases of OP toxicity are related to the inhibition of cholinesterase "enzymes" resulting in the accumulation of acetylcholine in the synaptic cleft, which induces an over stimulation of the autonomic nervous system throughout the body (De Candole et al., 1953; Tafuri and Roberts, 1987). Respiratory failure is considered the primary cause of death in acute OP poisonings (Durham and Hayes, 1962; Eddleston, Singh, et al., 2005; Lerman and Gutman, 1988). The mechanisms of OP-induced respiratory failure are thought to result from a direct depressant effect on the respiratory center in the brainstem, constriction of and increased secretion by the airways, and paralysis of the respiratory musculature (Bartholomew et al., 1985).

The standard therapeutic scheme of insecticide poisoning includes supportive treatment, antidote infusions (atropine and/or oximes), and decontamination (Lund and Monteagudo, 1986). Atropine competitively antagonizes accumulated acetylcholine at muscarinic receptors but is ineffective on nicotinic receptors (Durham and Hayes, 1962; Namba, 1972). Atropine is considered the first-line antidotal treatment to OP poisonings (Eddleston, Singh, et al., 2005, 2008; Houze et al., 2008; Villa et al., 2007). In contrast, the therapeutic use of oximes is controversial (Eddleston, Gunnell et al., 2005). Oximes, including pralidoxime and obidoxime, are potent cholinesterase reactivators that act by removing the phosphoryl group (Worek et al., 1997, 1999). In vitro experiments show that the efficiency...
of oximes is highly dependent on its concentration in the milieu (Eyer, 2003).

The dependence of oxime efficiency on plasma concentrations was previously suggested by Sundwall (1961). In anaesthetized cats poisoned with a sarin analog, N-methylpyridinium-2-dioxide methane sulfonate administration alone, without atropine, induced a more rapid improvement in hemodynamic and respiratory status with increasing dose levels and corresponding plasma drug concentrations (Sundwall, 1961). Attempts have been made to extend this finding to humans poisoned with OP insecticides, resulting in the development of various dosage regimens aimed at obtaining plasma pralidoxime concentrations at or above 4 mg/l but without definitive conclusions (Johnson et al., 1996; Medicis et al., 1996; Schexnayder et al., 1998; Willems et al., 1993). Only one prospective, randomized, open multicenter study showed a significant improvement in morbidity and mortality in moderate to severe poisonings with OP insecticides (Pawar et al., 2006). This study compared the efficiency of pralidoxime using a high-dose regimen (loading dose 2 g over 30 min, followed by the infusion of 1 g over 1 h, every hour for 48 h) versus the conventional dosage regimen (loading dose 2 g over 30 min, followed by the infusion of 1 g over 1 h every 4 h for 48 h). Thereafter, both groups received pralidoxime (1 g every 4 h) until patients were weaned off ventilators. Every patient was given 1.8–3.0 mg of atropine on admission, and then atropine was infused to correct muscarinic signs. Patients treated with the pralidoxime high-dose regimen significantly required less atropine during the first 24 h and less ventilatory support than the controls. This study supports the hypothesis that pralidoxime therapeutic effects are closely related to the pharmacokinetics (PK) of this drug. Unfortunately, plasma pralidoxime concentrations were not measured in this study.

The purpose of this study was to assess the concentration-dependent efficiency of pralidoxime on diethylparaoxon-induced respiratory toxicity in rats and the reactivation of whole-blood cholinesterase (WBChE). The relationship between diethylparaoxon concentrations and degree of inhibition of rat WBChE activity was evaluated in vitro, as well as the reactivation of cholinesterases by pralidoxime. For in vivo studies, the effects of pralidoxime exposure were assessed on ventilation at rest and in rats poisoned by diethylparaoxon (50% of LD50 or 0.215 mg/kg). A mechanism-based PK-toxicodynamic (PK-TD) model was developed that links in vitro and in vivo pharmacological effects and provides insights into the determinants of the complex temporal profile of pralidoxime reversal of diethylparaoxon-induced alteration of expiratory time, a major target of diethylparaoxon toxicity.

**MATERIALS AND METHODS**

All animal procedures used in this study were in strict accordance with the European Community Council Directive of 24 November 1986 (86-609/EEC) (protection of animals used for experimental and other scientific purposes) and Decree of 20 October 1987 (87-848/EEC).

**Animals.** Male Sprague-Dawley rats (Ifa-Credo, France) weighing between 200 and 250 g were used. Animals were allowed to acclimate for 1 week prior to experimentation in a temperature and light-controlled animal care unit. A standard diet and water was provided *ad libitum* for up to 1 day prior to study.

**Chemicals and study drugs.** Pralidoxime (CAS number: 6735-59-7) methylsulfate (Contratrinith) was a gift of SERB (Paris, France). Pralidoxime cation (PRX) was diluted in isotonic saline solution to obtain a 1.0 mg/ml mother solution, stored to –20°C, and stable during a maximum of 6 months. All doses are expressed as pralidoxime cation (PRX), and a correction factor of 1.7 between PRX and Contratrin can be used. Anesthetic drugs, ketamine (Ketalar) and xylazine (Rompum), were obtained from Parke Davis (Paris, France) and Bayer (Paris, France), respectively.

Diethylparaoxon in oil (diethyl-p-nitrophenyl phosphate, purity was greater than 90% CAS number: 311-45-5) (PO) was obtained from Sigma-Aldrich (St Quentin Fallavier, France). PO was diluted in dimethylsulfoxide so as to obtain a mother solution of 3.5 mg/ml. A daughter solution of PO was prepared in isotonic saline solution (140 mg/ml) to facilitate the injection of doses equal to 50% of the LD50. Solutions of PO were preserved at 4°C in the dark for a maximum period of 4 weeks. The stability of these aqueous solutions of diethylparaoxon was verified using high performance liquid chromatography (HPLC) with UV detection at 280 nm, as previously reported (Villa et al., 2007).

Guanosine, propionylthiocholine, Aldrihide (4, 4'-dithiodipyridine), 1-octane sulfonate sodium salt, dihydrated disodium phosphate, and monopotassium phosphate were obtained from Sigma-Aldrich. All others chemical reagents of analytical quality were obtained from Merck (Nogent-sur-Marne, France). Distilled water (Fre´senius FrancePharma, Louviers, France) was used for preparation of the various reagents.

**Pralidoxime reactivation of WBChEs in vitro.** The inhibition of WBChE was determined in rat whole blood according Augustinson et al. (1978). Complete method is described in WBChE reactivation Supplementary data.

**Pralidoxime PK in naïve and PO-poisoned rats.** Plasma pralidoxime PK were determined using an HPLC assay with electrochemical detection, as previously described (Houze et al., 2005). Complete method is described in pralidoxime PK Supplementary data.

**Pralidoxime effects at rest and in PO-poisoned rats.** Ventilation at rest was studied using whole-body plethysmography according to the barometric method described by Bartlett and Tenney (1970) with minor modifications (Houze et al., 2008; Villa et al., 2007). Complete method is described in plethysmography study Supplementary data.

**PO inactivation of in vitro WBChE.** The time-course of *in vitro* WBChE activity in rat whole blood after exposure to PO (10, 100, and 10^4 M) was modeled based on enzyme inactivation (Fig. 1). The rate of change of active enzyme (E_A) was defined by the following differential equation:

$$\frac{dE_A}{dt} = -\frac{k \cdot C_{PO}}{EC_{50,PO} + C_{PO}} \cdot E_A + k_i \cdot E_I$$

where \(k\) is the maximal rate constant of enzyme inactivation, \(C\) is the PO concentration, \(EC_{50,PO}\) is the concentration of PO that produces 50% of \(k\), \(k_i\) is a first-order reactivation rate constant, and \(E_I\) is the inactive enzyme pool. The PO concentration was assumed to be constant over the study period, and the initial condition of Equation 1 was set to 1 (i.e., 100%). The rate of change of the inactive enzyme was defined as:
Results are expressed as mean (±SEM). Model symbols are defined in “Materials and Methods” section. The initial condition of Equation 2 was set to 0 but was included in the final model as it improved predictive performance (data not shown). The initial condition of Equation 5 was set to 0 for im administration or to the administered dose for iv injection. The initial condition of Equation 6 was set to 0, and plasma drug concentrations (Cp) were modeled as: Cp = Ap/Vc, where Vc is the volume of the central compartment.

**PRX reactivation of in vitro WBChE.** Pridloidoxine reactivation of in vitro WBChE activity in rat plasma after 30 min of PO exposure (10nM) was described using an indirect response model. Equations 1 and 2 were modified to include simulation of the reactivation rate constant, kr, by PRX concentrations (CPRX):

\[
\frac{dE_1}{dt} = \frac{k \cdot C_{PO}}{EC_{50,PO} + C_{PO}} \cdot E_A - (k_t + k_{age}) \cdot E_1
\]  

(2)

where \(k_{age}\) is a first-order rate constant of aging of inactive enzyme. Initially, \(k_{age}\) was set to 0 but was included in the final model as it improved predictive performance (data not shown). The initial condition of Equation 2 was set to 0.

**PRX pharmacokinetics.** All mean plasma concentration-time profiles were fitted simultaneously using a standard linear two-compartment model (as shown in Fig. 3b):

\[
\begin{align*}
\frac{dA_p}{dt} &= k_4 \cdot D_{IM} \cdot e^{-kt} - (k_{12} + k_0) \cdot A_p + k_{21} \cdot A_t \quad (5) \\
\frac{dA_t}{dt} &= k_{12} \cdot A_p - k_{21} \cdot A_t \quad (6)
\end{align*}
\]

where \(A_p\) and \(A_t\) represent the amounts of PRX in the central and peripheral compartments, \(k_4\) is a first-order absorption rate constant, \(D_{IM}\) is the im dose, \(t_{lag}\) is the temporal delay in the absorption of drug after im injection, \(k_{12}\) and \(k_{21}\) are first-order rate constants of drug distribution between the central and peripheral compartments, and \(k_0\) is a first-order elimination rate constant. The initial condition of Equation 5 was set to 0 for im administration or to the administered dose for iv injection. The initial condition of Equation 6 was set to 0, and plasma drug concentrations (Cp) were modeled as: \(C_p = A_p/V_c\), where \(V_c\) is the volume of the central compartment.

**PRX in vivo TD.** For in vivo dynamics, plasma concentrations of PO were not available. Therefore, an empirical kinetic function was used to define the inactivation of WBChE activity:

\[
\begin{align*}
k_{PO}(t) &= k_p \cdot \alpha \cdot t \cdot e^{-k_t \cdot t} \\
\end{align*}
\]

(7)

with \(\alpha\) representing the magnitude of the function and \(k_p\) as a first-order formation and dissipation rate constant. Hence, the rates of change of active and inactive enzyme were defined as:

\[
\begin{align*}
\frac{dE_A}{dt} &= -k_{PO}(t) \cdot E_A + k_t \cdot \left(1 + \frac{E_{max} \cdot C_{PRX}}{EC_{50,PRX} + C_{PRX}}\right) \cdot E_1 \\
\frac{dE_1}{dt} &= k_{PO}(t) \cdot E_A - k_{age} \cdot E_1
\end{align*}
\]

(8)

(9)

Plasma PRX concentrations (Cp) after a 50 mg/kg im injection were described by Equations 5 and 6, and the PK parameters were fixed. An additional 30 min was added to \(t_{lag}\) to mimic the study conditions of injecting PRX 30 min after PO exposure. The parameters \(k_t, E_{max}, EC_{50,PRX}, h, \) and \(k_{age}\) were fixed to values estimated from the in vivo analysis (Equations 3 and 4). The TD biomarker, expiratory time (TE), was linked to active enzyme \(E_A\) according to the following nonlinear transfer function:

\[
T_E = T_E^0 + \frac{E_{max,T_E} \cdot \left(\frac{E_A}{E_{max,T_E}} - 1\right)^n}{E_{max,T_E} + \left(\frac{E_A}{E_{max,T_E}} - 1\right)^n}
\]

(10)

where \(T_E^0\) is the baseline expiratory time (fixed to 0.37 s), \(E_{max,T_E}\) is the maximal increase in \(T_E\), \(E_0\) is the baseline active enzyme (1 or 100%), \(E_{50}\) is the corrected enzyme ratio resulting in 50% of \(E_{max,T_E}\), and \(n\) is a sigmoidicity coefficient (fixed to 5). Thus, under baseline conditions, \(E_0/E_{max,T_E} = 1\) and \(T_E = T_E^0\). Enzyme inactivation results in a decrease of \(E_A\), \(E_0/E_{max,T_E}\) will become greater than 1, and as this ratio becomes large relative to the \(E_{50}\), \(T_E \rightarrow T_E^2 + E_{max,T_E}\). The final set of parameters remaining to be estimated from fitting Equations 7–10 to the time-course of \(T_E\) included \(E_0, k_p, E_{max,T_E}\), and \(E_{50}\).

**Data analysis.** All model fitting/parameter estimation was conducted using the maximum likelihood estimator in ADAPT II (Biomedical Simulation...
Resource, University of California, Los Angeles, CA). A standard variance model was specified:

$$\text{VAR} = \sigma_i^2 + \nu^2$$

with \( Y \) representing model predicted values, and separate variance model parameters (\( \sigma_i \)) were estimated for the analysis of (1) \textit{in vitro} WBChE inactivation, (2) \textit{in vitro} WBChE reactivation by PRX, and (3) \textit{in vivo} PRX effects on \( T_E \). Model development was guided by the Akaike Information Criterion, Schwarz Criterion, program convergence, precision of parameter estimates, distribution of residuals, and visual inspection of temporal profiles.

Statistical analysis. All measurements and calculations were expressed as mean ± SEM. Global PK parameters were initially determined using noncompartmental methods in WinNonlin (Pharsight Corporation, Mountain View, CA). The Kruskall-Wallis ANOVA followed by multiple Dunn’s comparison tests was used to compare the global PK parameters, ventilation at rest measured using plethysmography, and WBChE activities. For each animal and ventilation parameter, we calculated the difference between the value at each sampling time and its corresponding baseline value at that time. All ventilation parameter tests were two tailed and performed using Prism version 5.0 (GraphPad Software, Inc., San Diego, CA). A \( p \) value of less than 0.05 was considered significant.

RESULTS

\textbf{WBChE In Vitro Inactivation by PO and Reactivation with PRX}

Blood specimens were collected from male Sprague-Dawley rats. The inhibition of WBChE was induced by three concentrations of PO (10, 100, and \( 10^3 \)nM) expressed as residual activity (Fig. 1). The \( 10^3 \)nM induced a complete, rapid (< 1 min), decrease of WBChE to less than 10%, plateauing during the study period. The 100nM concentration resulted in the same level of residual activity, albeit after 20 min post–PO exposure. The 10nM concentration induced a progressive decrease over 30 min after PO addition to a residual activity of 30%.

A simple enzyme inactivation model reasonably described the decrease in rat WBChE activity over 30 min following PO exposure (Fig. 1). Final parameters are listed in Table 1 and were estimated with good precision. A maximum rate constant of enzyme inactivation (\( k \)) was estimated to be 3.13/min, and the concentration of PO producing 50% of the maximal rate of inactivation (EC50,PO) was estimated to be 75.9nM. An initial model excluded a first-order rate constant of aging of inactive enzyme (\( k_{age} \)); however, this parameter improved model fitting criteria and was estimated to be relatively slow as compared with the rate of enzyme reactivation (\( k_r \)) (0.0226 vs. 0.310/min).

The \textit{in vitro} efficiency of PRX to reactivate WBChE activity was studied at a fixed PO concentration of 10nM. We tested six concentrations of PRX ranging from 0 to 10 mg/l. A log-linear correlation between the area under the curve (AUC) of reactivation curves shown in (A) as a function of PRX concentrations. All results are expressed as mean ± SEM (\( n = 3 \)).

![FIG. 2. Antidotal activity of pralidoxime on WBChE reactivation.](image)

(A) Residual activity of WBChE following exposure to PO (10nM) and various concentrations of PRX introduced 30 min after treatment with PO (○, 10; ▼, 6; ▲, 4; ■, 2; ●, 1; and □, 0 mg/l). Open and closed octagons represent control and PRX treatment in absence of PO. Lines represent model-fitted profiles according to a modified enzyme inactivation model (Equations 3 and 4). (B) Area under the curve (AUC) of reactivation curves shown in (A) as a function of PRX concentrations.

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>PO (CV%)</th>
<th>PO + PRX (CV%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k ) (1/min)</td>
<td>3.13 (5.9)</td>
<td>2.52 (15)</td>
</tr>
<tr>
<td>EC50 (nM)</td>
<td>75.9 (5.3)</td>
<td>75.9 (—)</td>
</tr>
<tr>
<td>( k_r ) (1/min)</td>
<td>0.310 (11)</td>
<td>0.111</td>
</tr>
<tr>
<td>( k_{age} ) (1/min)</td>
<td>0.0226 (13)</td>
<td>0.00188</td>
</tr>
<tr>
<td>( E_{max} )</td>
<td>N/A</td>
<td>3.47 (14)</td>
</tr>
<tr>
<td>( EC_{50,PRX} ) (mg/l)</td>
<td>N/A</td>
<td>4.67 (9.5)</td>
</tr>
<tr>
<td>( H )</td>
<td>N/A</td>
<td>3.15 (10)</td>
</tr>
</tbody>
</table>

Note. CV = coefficient of variation; N/A, not applicable.

\*Fixed value.
The enzyme inactivation model (Equations 1 and 2) was modified to include PRX stimulation of enzyme reactivation (Equations 3 and 4), and the model well captured the time-course of WBChE activity resulting from all treatment conditions (Fig. 2A). The final estimated parameters are also listed in Table 1. Given the relatively longer duration of the reactivation study, the rate constants of enzyme inactivation, reactivation, and aging of inactive enzyme were reestimated. Whereas the final values for \( k \) and \( k_r \) were of the same order of magnitude, the estimated value of \( k_{age} \) was much lower than the previous estimate from the short duration inactivation study (0.00188 vs. 0.0226/min). Because only one concentration of PO was evaluated in the enzyme reactivation analysis (10nM), the EC_{50,PO} was fixed to 75.9nM. The maximal fold increase in \( k_r \) induced by PRX (\( E_{max} \)) was estimated to be 3.47, and the Hill coefficient (h) was 3.15, suggesting a steep concentration-effect relationship. The PRX concentration producing 50% of maximal stimulation (EC_{50,PRX}) was estimated to be 4.67 mg/l.

PRX PK in Naïve and PO-Poisoned Rats

After iv bolus administration for all dose levels, plasma concentrations of PRX decreased rapidly with short elimination half-lives (Fig. 3A). For the 50 mg/kg dose, plasma PRX concentrations decreased below the 4 mg/l target plasma concentration in less than 30 min after injection (Fig. 3A). A linear two-compartment model with linear first-order absorption is shown in Figure 3B, and a brief absorption phase was observed after im PRX administration in naïve rats (Fig. 3C). The maximal measured concentrations (\( C_{max} \)) were observed 10 min after injection and were proportional to the injected doses (\( R^2 = 0.993, n = 5 \)). The mean bioavailability was 89 ± 5, 98 ± 3, and 99 ± 2% for the 10, 25, and 50 mg/kg dose levels. The im administration of PRX (50 mg/kg) resulted in plasma PRX concentrations above 4 mg/l for about 50 min postinjection (Fig. 3C).

Table 2 shows the global PK parameters of PRX administered at the three doses by the two routes in naïve rats. For the same route, the half-lives did not show any
significant differences between the dose levels. The PK profiles of the three doses administered im showed a significantly slower decline of PRX plasma concentrations in comparison with the three doses administered by the iv route. The elimination half-lives were significantly longer after im compared with the iv injection ($p < 0.01$). The area under curves (AUCs) were not significantly different for the two routes and were proportional to the injected doses ($R^2 = 0.992$ and 0.987 for iv and im doses). No significant differences were observed regarding the total clearances and volumes of distribution for the two routes at the same dose.

We compared the PK of a single dose of PRX (50 mg/Kg) administered im with control rats receiving the vehicle of PO to that in PO-poisoned rats (Fig. 3D). The $C_{\text{max}}$ values were not significantly different in the poisoned rats compared with the control rats. Thereafter, the plasma PRX concentrations remained significantly greater in poisoned rats compared with control rats from 60 to 180 min postinjection. PO pretreatment slightly but significantly increased the elimination half-lives from 54 ± 9.3 to 62 ± 4 min in control and PO-poisoned rats, respectively. PO pretreatment slightly but significantly increased the AUCs by 12% ($p < 0.01$) in PO-poisoned rats in comparison with control rats. PO pretreatment significantly decreased the total body clearance of 28% in PO-poisoned rats in comparison with control rats without significant modification of the volume of distribution (Table 2).

The plasma PRX concentrations following iv and im administration were well described by the model (Fig. 3B) and fitted profiles are shown in Figure 3. In order to capture changes in PK after PO exposure, several model parameters were tested and allowed to differ between control and PO-treated animals. Although separate first-order rate constants of elimination ($k_{el}$) and distribution from the central to peripheral compartment ($k_{12}$) each improved model fitting criteria, allowing $k_{12}$ to differ between groups provided the best results.

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Estimate values (CV%) All rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_a$ (/min)</td>
<td>0.0702 (13)</td>
</tr>
<tr>
<td>$t_{lag}$ (min)</td>
<td>1.80 (15)</td>
</tr>
<tr>
<td>$k_{el}$ (/min)</td>
<td>0.160 (12)</td>
</tr>
<tr>
<td>$k_{12N}$ (/min)</td>
<td>0.125 (16)</td>
</tr>
<tr>
<td>$k_{12P}$ (/min)</td>
<td>0.301 (25)</td>
</tr>
<tr>
<td>$k_{31}$ (min)</td>
<td>0.0337 (11)</td>
</tr>
<tr>
<td>$V_d$ (l/Kg)</td>
<td>0.454 (13)</td>
</tr>
</tbody>
</table>

The $k_{12}$ distribution parameters were estimated as 0.125 and 0.301/min in control and PO-treated rats, whereas all other model parameters were shared between the two groups (Table 3). A bioavailability parameter was initially included to allow for incomplete absorption after im administration. However, the 95% confidence interval for this term was estimated to be 0.922–1.22 and was fixed to 1.0 (i.e., complete bioavailability) in the final analysis. Despite slight systematic deviations in the terminal phase of the profiles for the 10 and 25 mg/kg iv dose levels, the data were well described overall and model parameters were estimated with low coefficient of variation supporting the assumption of good precision. This PK study showed that the 50 mg/kg im dosage regimen resulted in plasma PRX concentrations above the empirical target of 4 mg/l for the longest period of time for the range of tested doses in PO-poisoned rats. Accordingly, this dosage regimen was selected to determine the efficiency of PRX as an antidote in PO-poisoned rats.

**PRX Effects on Respiration and WBChE at Rest and in PO-Poisoned Rats**

No significant differences were observed in respiratory parameter baseline values between control and PRX groups.

**TABLE 2**

Noncompartmental PK Parameters of Pralidoxime in Naïve and PO-Poisoned Rats

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Pralidoxime iv route</th>
<th>Pralidoxime im route</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{\text{max}}$ (min)</td>
<td>10 mg/Kg (n = 5)</td>
<td>25 mg/Kg (n = 5)</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (mg/l)</td>
<td>8.3 ± 2.4</td>
<td>55.1 ± 8.9</td>
</tr>
<tr>
<td>Alpha</td>
<td>3.6 ± 0.7</td>
<td>3.8 ± 0.7</td>
</tr>
<tr>
<td>Beta</td>
<td>26.6 ± 2.8</td>
<td>28.2 ± 2.7</td>
</tr>
<tr>
<td>AUC (mg/min/l)</td>
<td>118.8 ± 23.5*</td>
<td>359.7 ± 30.3</td>
</tr>
<tr>
<td>Cl (l/kg/h)</td>
<td>5.18 ± 0.75</td>
<td>4.28 ± 0.38</td>
</tr>
<tr>
<td>Vd (l/Kg)</td>
<td>3.34 ± 0.89</td>
<td>2.92 ± 0.77</td>
</tr>
</tbody>
</table>

Note. All results are expressed as mean ± SEM. Statistical significance: iv route: *$p < 0.01$ versus 25 mg/kg; im route: †$p < 0.01$ versus 25 mg/kg; §$p < 0.01$ versus 50 mg/kg; p < 0.01 im vs iv route.
After PO administration, there was a significant decrease in respiratory frequency \( (f) \) from 121 ± 8 b/min at baseline to 72 ± 2 b/min 30 min after PO injection (Fig. 4). Conversely, there was a significant increase in the total time of a respiratory cycle \( (T_{TOT}) \) from 0.47 ± 0.02 s at baseline to 0.87 ± 0.02 s 30 min after PO injection. Thereafter, the decrease in \( f \) and the increase in \( T_{TOT} \) values plateaued and remained significantly different from the values in the control group until the end of the study. The increase in \( T_{TOT} \) resulted from an increase in expiratory time \( (T_E) \) with no significant effect on the inspiratory time \( (T_I) \).
In contrast, PO-poisoned rats treated with PRX 30 min postinjection of PO exhibited a significant reactivation of cholinesterase activity, increasing to 70 and 95% of residual activity at 30 and 60 min after PRX administration. Thereafter, there was a nonsignificant decrease of WBChE activity from 95 to 90% at 60 and 180 min after PRX administration, respectively (Fig. 5).

As there was a marked effect of PO on $T_E$, the PK-TD relationship was initially studied by plotting $T_E$ values against the corresponding plasma PRX concentrations. The PK-TD profile showed a counterclockwise hysteresis or temporal disconnect (Fig. 6A). During the absorption phase, 7 mg/l of PRX was associated with an improvement of $T_E$ values, whereas the same concentration during the elimination phase was associated with a nearly complete rebound in respiratory toxicity. The increase of plasma PRX concentrations from 0 to 9 mg/l was associated with an improvement of $T_E$ values. The improvement of $T_E$ values extended up to concentrations of up to 9 mg/l. At this concentration, the $T_E$ nearly returned within the normal range. Thereafter, within the range of 9–14 mg/l, there was no additional improvement in $T_E$ values. During the decrease in plasma PRX concentrations, there was a rapid increase in $T_E$ values, returning at the values observed in PO-poisoned rats, corresponding to an increase of $T_E$ at 200% of the basal value. The onset of increase in $T_E$ values occurred well before PRX concentrations decreased below the theoretical target of 4 mg/l. Indeed, the $T_E$ values returned nearly to those observed in the PO group at plasma PRX concentration of about 11 mg/l.

A mechanism-based pharmacodynamic model was developed to describe the time-course of expiratory time ($T_E$) in rats after PO exposure (0.215 mg/kg, sc) and subsequent PRX administration of 50 mg/kg im (Fig. 6B). Starting with the in vitro WBChE reactivation model (Equations 3 and 4), concentrations of PRX were replaced by the PK of the drug after intramuscular administration in rats after PO exposure. This profile was fixed as a known driving function using the standard two-compartment model and the final parameter estimates reported in Table 3. Because the PK of PO was not determined, an empirical function was used to mimic the time-course of enzyme inactivation (Equation 7). Finally, pilot data suggested that the change in enzyme activity relative to the initial value ($1/E_\lambda$) could be linked to expiratory time using a nonlinear transfer function or Hill-type relationship (Equation 10, data not shown). The values for $k_r$, $k_{age}$, $E_{max}$, $EC_{50,PRX}$, and $h$ were all fixed to estimates obtained from the in vitro enzyme reactivation analysis (Table 1).

The time-courses of expiratory time ($T_E$) were well described by the final TD model and fitted profiles are shown in Figures 6C and 6D. Final estimated parameter values are listed in Table 4. The empirical enzyme inactivation function was sufficient to lower $E_{\lambda}$ and produce a sustained increase in $T_E$ from baseline (0.37 s) to 0.67 s in rats not treated with PRX. The first-order rate constant of the inactivation function was estimated to be
relatively slow (0.00798/min), suggesting a sustained exposure to PO after sc administration. The capacity ($E_{\text{max}}$, $T_E$) and sensitivity ($E_{50}$, $T_E$) parameters of the nonlinear transfer function were estimated to be 0.289 s and 0.922 (unitless term), and the Hill coefficient of this relationship ($n$) was fixed to 5, reflecting a steep sigmoidal curve. The transient rescue in $T_E$ from PRX exposure was also well captured by the final model (Fig. 6D).

**DISCUSSION**

Our results of PO-induced inactivation of WBChE in vitro are in agreement with those previously reported by Worek et al. (1997), in which residual activity of human erythrocyte cholinesterase 30 min after the addition of PO (160nM) was about 10%. We chose the 0.01 mM concentration of PO to study the concentration-dependent reactivation by PRX, based on a previous study dealing with PO-induced respiratory toxicity in rats (Villa et al., 2007). The efficiency of oxime reactivation of cholinesterase activity is concentration dependent (Worek et al., 1997, 1999). We also observed a log-linear correlation between PRX concentrations and WBChE reactivation. Even for the highest tested concentration (10 mg/l), no saturation of the reactivation process was observed. This is in agreement with Eyer’s (2003) suggestion that 100µM of obidoxime (13.7 mg/l) should be targeted to optimize cholinesterase reactivation. Within the range of studied PRX concentrations, no limitation for cholinesterase reactivation was observed. Despite in vitro support for oxime reactivation of cholinesterase activity, clinical efficiency remains controversial.

The administration of PRX (5 mg/kg) was associated with a partial improvement in sarin-poisoned cats, plasma concentrations ranged from 2 to 4 mg/l. The administration of a 10 mg/kg dose of PRX was associated with a normalization of signs and symptoms, plasma concentrations ranged from 4 to 12 mg/l (Sundwall, 1961), suggesting that plasma PRX concentrations above 4 mg/l counteracted sarin toxicity. This finding has been extended to humans poisoned with OPs,
resulting in the development of dosage regimens attempting to maintain plasma PRX concentrations at or above 4 mg/l (Medics et al., 1996; Schexnayder et al., 1998; Willems et al., 1992). Interestingly, the PRX concentration producing 50% of maximal stimulation of WBChE reactivation (EC50,PRx) was estimated to be 4.67 mg/l, which agrees well with the in vivo target concentration of 4 mg/l.

Our PK data after im administration favorably compared with the literature (Green et al., 1986; Simons and Briggs, 1983). Kinetic differences have been reported for healthy and poisoned patients with elimination half-lives correlating with the enhancement of the volume of distribution. The approximate 2.5-fold increase in the first-order distribution rate constant (k12) results in an increase in the steady-state volume of distribution (Vss): Vss = Vc(1 + k12/k21). Hence, Vss increases from 2.14 to 4.51 l/kg in poisoned rats. Given that PRX exhibits very little binding to plasma proteins (Eyé, 2003), a decrease in drug-free fraction in tissue might represent one mechanism by which the Vss increases in rats exposed to PO poisoning. One hypothesis is that enough drug might be bound to the pharmacological target (inactive enzyme) to influence PRX PK, so-called target-mediated drug disposition, which has been observed for other drug-enzyme systems (Levy, 1994; Mager, 2006).

The apparent ineffectiveness of oximes for OP poisoning might be explained by PK mechanisms, including (Eyé, 2003) (1) insufficient oxime dosage relative to the degree of OP exposure, (2) optimal oxime concentration not maintained for long enough duration, and (3) reinhibition of the enzymes by sustained or high levels of OP and OP persistence in deep compartments. For the in vivo studies, the molar ratio between PO and PRX was 1:2000, bioavailability of im PRX was nearly complete, and the 50 mg/kg dose resulted in drug concentrations exceeding 4 mg/l. Nevertheless, there was a rebound in respiratory toxicity, whereas plasma PRX was still around 11 mg/l during the elimination phase. Furthermore, the rebound was not associated with decreasing cholinesterase activities in various tissues (data not shown) and blood. The near normalization of WBChE activities over the 210-min period after PRX injection does not suggest enzyme reinhibition by OP. Notwithstanding the short elimination half-life of PRX, a PK mechanism alone cannot account for the lack of sustained effect of PRX.

The efficiency of PRX on respiratory function in awake animals has been poorly studied. For the first time, we describe a PK-TD relationship (counterclockwise hysteresis profile) between plasma PRX concentrations and respiratory time variations. The decrease of expiratory time was observed with plasma PRX concentrations increasing from 0 up to 9 mg/l (i.e., for concentrations near the upper theoretical value). This suggests that the effect of pralidoxime is directly proportional to plasma concentrations (Cavaliere et al., 1998; Newman, 2004). Worek et al. (1997) proposed that increased plasma obidoxime concentrations (between 10 and 15 mg/l) might result in prolonged antidotal activity. Our study has shown a steady state for pralidoxime efficiency (between 9 and 12 mg/l), suggesting that higher PRX concentrations are unnecessary.

The final TD model of PO and PRX effects on Tₑ integrates in vitro enzyme kinetics with other factors influencing the time-course of in vivo drug effects. Coupling with the in vitro data was essential, as many of the system and drug-specific parameters were not identifiable from the Tₑ profiles and single-dose study design exclusively. Modeling in vitro enzyme inactivation and reactivation provided key system rate constants as well as the capacity (Emax) and potency (EC50) of PRX effects, the latter of which agreeing with prior empirical evidence (Sundwall, 1961). The enzyme inactivation model resembles others in the literature, including chlorpyrifos toxicity (Timchalk et al., 2002), but excludes turnover processes of active enzyme synthesis and degradation, given the relatively short study duration. The effect of stimulating the first-order rate constant of enzyme reactivation or regeneration is consistent with precursor-dependent indirect models (Sharma et al., 1998) and the mechanism of PRX action. Such models accommodate concentration-effect hysteresis. Model simulated profiles of PRX concentrations and (1/Eₐ) are shown in Figure 7A. In the absence of PRX, the empirical inactivation function results in an increase in the inverse of Eₐ from baseline conditions (1.0) to about 7 after PO treatment. The sharp relationship between (1/Eₐ − 1) and Tₑ (Fig. 7B) results in peak values of Tₑ for any value of 1/Eₐ greater than 3.0, which occurs around 30 min after PO treatment. The im administration of PRX results in drug concentrations above the EC50 (4.67 mg/l) and almost completely reverses the increase in 1/Eₐ. However, as concentrations decrease toward the EC50, 1/Eₐ begins to increase (even for concentrations above the EC50) and is back to pretreatment values (around 3.0) by 100 min after PO exposure or 70 min after PRX administration. Therefore, the PRX concentration-effect hysteresis (Fig. 6A) can be readily explained by the indirect nature of drug action and a steep relationship between enzyme activity and expiratory time.

In our study, we have tested only one pesticide, PO, with a limited dose to 50% of the LD₅₀. However, this dose induces significant respiratory effects in comparison with controls.
Respiratory effects after a higher dose (75% of the LD50) were not significantly different (Villa et al., 2007). Cavaliere et al. (1998) used doses of four OPs at or above 50% of the LD50. Formation of phosphoryloximes during the reactivation process may paradoxically inhibit AChE to a greater extent and longer duration than would be present without oxime therapy. However, this is more significant with obidoxime than PRX (Aaron, 2007). Oxime-induced reactivation of acetylcholinesterase inhibited with pesticides should be less complicated than with nerve agents, owing to the rapid decomposition of O,O-diethylphosphoryloximes compared with O-alkyl methylphosphonyl pyridine oximes and N,N-dimethylamidophosphonyl pyridine oximes (Ashani et al., 2003). Our use of diethylparaoxon should yield rapidly degrading diethylphosphoryloximes. However, there were significant improvements in cholinesterase activities in various tissues, including the blood. The range in residual activities reported here is not associated with clinical disturbances and thus does not support phosphoryloxime yield as an explanation for the rebound of respiratory toxicity.

WBChE basal in vivo activity was similar to a previous study describing a rat model of diethylparaoxon poisoning (Villa et al., 2007). We showed a rapid and prolonged reactivation of WBChE after pralidoxime administration, suggesting that the antidotal activity of pralidoxime was significantly different on respiratory function and cholinesterase reactivation. Several hypotheses may explain this discrepancy: (1) oxime can reactivate cholinesterase anywhere (Benshop et al., 1976); however, some studies have reported that the degree of reactivation is dependent on the nature and location of the synapse (Besser et al., 1995; Finkelstein et al., 1988); (2) oxime efficiency may be OPs and species dependent (Eyer, 2003); and (3) the measurement of cholinesterase activity is not closely related to treatment efficiency (Aygun et al., 2002; Balali-Mood and Shariat, 1998; Besser et al., 1995; Finkelstein et al., 1988). Neumuscular transmission might represent a better criterion to evaluate oxime antidotal activity than cholinesterase reactivation (Besser et al., 1995). Others have also reported a disconnect between cholinesterase reactivation and oxime protective effects (Aygun et al., 2002; Eddleston et al., 2002). Polyzou et al. (1998) demonstrated that the mortality of PO-poisoned honeybees is not because of a lack of cholinesterase reactivation. This suggests that OP may have secondary targets of toxicological importance on which PRX is perhaps inefficient (Pope and Liu, 2006). Our study suffers from a number of limitations. First, our study results from the measurement of PRX concentrations only in plasma and not in relevant tissues, including the brain. However, Sakurada et al. (2003), using brain microdialysis technique with HPLC UV, reported that the striatal extracellular/blood concentration ratio 1 h after the iv injection of a 50 mg/kg dose of 2-PAM in the Wistar rats was 0.093 ± 0.053. Second limitation resulted from the fact we assessed the efficiency of PRX only 30 min after PO injection. Further studies are required to assess the efficiency of PRX in earlier as well as later times of injection than 30 min post-PO. The third limitation of our study resulted from the use of a single PO concentration (10nM) when evaluating PRX efficiency in the in vitro study. Based on our estimated EC50 value (75nM), it would be of interest to test PRX antidotal effect at PO concentrations near the EC50 value. Because WBCHe activity was measured without a butyrylcholinesterase inhibitor, caution is needed regarding extension of our results collected in rats to humans, given the considerable species difference in butyrylcholinesterase levels.

In conclusion, PRX PK depends on the poisoned status of the animal. PRX antidotal efficiency in vitro is strongly correlated to its concentration. In PO-poisoned rats, we
observed a discrepancy between reactivation of WBChE activity and respiratory effects. Our results suggest that cholinesterase reactivation is a necessary but not sufficient condition to account for the antidotal effect of pralidoxime.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

SERB Laboratories (Paris, France).

ACKNOWLEDGMENTS

We are indebted to Mrs Claire Monier for her valuable technical assistance. Mrs Monier passed away on the eighth of January 2007. SERB laboratories did not have any control over the resulting publication. The authors declare no conflict of interest.

REFERENCES


