Comparative Study of Continuous Pralidoxime Infusion versus Intermittent Dosing: Application of High-Performance Liquid Chromatography Method on Serum of Organophosphate Poisoned Patients

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Abstract

Background: The effective therapeutic dose of pralidoxime methylsulphate for organophosphate (OP) poisoning is necessary to be clarified. This study was designed to comparatively assess the blood level of pralidoxime (BPL) and clinical outcomes in OP poisoned patients treated with intermittent dosing and patients treated with continuous infusion.

Methods: This was a prospective, open labelled, cross-sectional, nonrandomized observational study which was done from 2009 to 2012 in a tertiary care hospital in Manipal, India. A high-performance liquid chromatography (HPLC) method with prominence diode array (PDA) detector was developed to measure BPL. Patients were categorized into study and control groups. Patients in study group were divided into 3 subgroups as they were treated with (a) intermittent pralidoxime dosing (1 g/q8h) or (b) continuous pralidoxime infusion (500 mg/h) or (c) continuous pralidoxime infusion (1 g/h). Patients who were not treated with pralidoxime were considered as the control group. The level of acetylcholinesterase (AChE) was measured before and pralidoxime therapy.

Results: The developed HPLC method was linear over the range of 0.5-50 µg/mL and the correlation coefficient was found to be greater than 0.99. The median (IQR) of BPL in intermittent dosing (4.63 (5.26)) was comparatively lower than patients treated with continuous infusion. The highest BPL was maintained in 1 g/h group with median (IQR) serum level of 38.86 (16.75). The reactivation rate of AChE was higher in continuous infusion groups compared to intermittent dosing. Comparison of AChE before and after pralidoxime therapy showed that higher BPL was associated with greater reactivation of AChE.

Conclusion: HPLC can be used as alternative method for measurement of pralidoxime level in blood. Continuous infusion of pralidoxime maintained a steady higher blood concentration compared to intermittent dosing with vast fluctuations. The reactivation rate of AChE was higher in continuous infusion compared to intermittent dosing. Hence, continuous infusion of pralidoxime can more rapidly recover the OP poisoned patients with less morbidity.

Keywords: High Performance Liquid Chromatography; Organophosphate Poisoning; Pralidoxime Compounds

INTRODUCTION

Organophosphate (OP) pesticide poisoning is a major public health problem in developing countries such as India, Bangladesh and Sri Lanka (1-3). World Health Organization estimated that OP pesticides are responsible for around 200,000 deaths per year (1). Early identification followed by effective management in the initial stages increases the rate of survival among OP poisoned patients. Standard treatments include intravenous administration of atropine and pralidoxime to counteract the acetylcholinesterase (AChE) inhibition (4). The role of atropine in treatment of OP poisoning is already well established (4). Use of oximes in OP poisoning is still controversial and varies in opinion among physicians with regards to dose, regimen and duration of treatment. There is a very little evidence available to prove the importance of oximes in treatment of acute OP poisoning (5). Blood level of pralidoxime (BPL) plays an important role in reactivation of AChE in OP poisoned patients. Most of the studies showed the minimum blood level of pralidoxime required to reactivate AChE is 4 µg/mL with a range of 4-12 µg/mL (6,7). A study by Jovanovic et al. on OP poisoned patients demonstrated that cholinesterase reactivation in human cases of OP poisoning treated with pralidoxime methylsulphate, not only depends on the plasma concentration of the oxime, but also on the plasma concentration of the OP agent (8). Their study also showed that oxime concentrations of about 4 mg/L is effective if the plasma concentrations of OP compounds were below 50 µg/L (8).

It has been shown that intermittent bolus dose of pralidoxime is associated with higher BPL in the initial stages followed by very low concentrations over the period of time (9-11). Continuous infusion of pralidoxime can maintain constant blood level with no significant adverse
effects (9,11). However, BPL in OP poisoned patients varies from patient to patient, and no clinical studies are available in the literature to compare the blood level of pralidoxime in various dose regimens with their outcome. There are only few studies available which described the measurement of BPL in the OP poisoned patients (11-15). This study was designed to comparatively assess the BPL in OP poisoned patients treated with intermittent dosing and patients treated with continuous infusion using the high-performance liquid chromatography (HPLC) method with prominence diode array (PDA) detector. Also, it was aimed to compare clinical outcomes among different dose regimens.

**METHODS**

**Study design**

This was a prospective, open labelled, cross-sectional, nonrandomized observational study which was conducted from February 2009 to November 2012 in a tertiary care hospital in Manipal, India. The study received ethical committee approval of Manipal College of Pharmaceutical Sciences.

**Study population and inclusion criteria**

All OP poisoned patients admitted to emergency department of Kasturba Hospital, Manipal, irrespective of age and sex were included in the study. Patients who concomitantly ingested carbamates, organochlorines or acetanilide derivatives and those with chronic poisoning were excluded. Informed consent according to the Helsinki declaration was taken from each patient. Patients were categorized into study and control groups. Patients in study group were divided into 3 subgroups as they were treated with (a) intermittent pralidoxime dosing (1 g/q8h) or (b) continuous pralidoxime infusion (500 mg/h) or (c) continuous pralidoxime infusion (1 g/h). Patients who were not treated with pralidoxime were considered as the control group.

**Development of HPLC method for measurement of blood level of pralidoxime**

**-Reagents used:** Methanol and acetonitrile (HPLC grade) were obtained from Lab-Scan., Mumbai, India. Trichloroacetic acid (20%) (AR Grade) was obtained from S.D. fine chem. Ltd, Mumbai, India. Glacial acetic acid (AR Grade) was purchased from Qualigen Fine Chemicals, Mumbai, India. Pralidoxime methylsulfate was purchased from Sigma-Aldrich Chemie, Steinheim, Germany. Water for injection (HPLC Grade and Milli-Q, Millipore) was used for preparation of different reagents.

**-Chromatographic conditions:** The method applied was a reversed phase- HPLC technique and Isocratic mode of gradient technique. Separation was done using a 5µm Hypersil Base Deactivated Silica (BDS) Column (250x4.6 mm). The mobile phase (0.2%) was composed of glacial acetic acid: acetonitrile (92:8 v/v). A PDA was used to detect pralidoxime levels with the detection wavelength set at 294 nm. The flow rate was maintained at 1.2 mL/min. The column oven temperature was set at 250°C while the autosampler temperature was set at 50°C. Oxcarbamazepine (1 µg/mL) was used as an internal standard (IS) as it has the similar physicochemical properties including solubility and wavelength to pralidoxime.

**-Biological sample collection, processing and storage:** Four-millilitre blood sample was collected at the 24th hour of starting pralidoxime. From patients treated with continuous infusion of pralidoxime one sample was collected. From intermediate dosing group two samples were collected, one at peak concentration and another at trough. The peak sample was collected after half an hour of administration of pralidoxime and trough sample was collected just before repeating the dose of pralidoxime. The blood samples were centrifuged at 3500 rpm for 5 minutes at 4°C and the serum was separated and stored in deep freezer at -70°C until further analysis.

**-Extraction technique:** For extraction of pralidoxime from blood samples, protein precipitation method was used. Protein precipitation solvents included acetonitrile, methanol, trichloroacetic acid and perchloroacetic acid. Based on the extraction efficiency and selectivity parameters, 20% trichloroacetic acid (TCA) was selected as the extracting solvent.

Pralidoxime stock solutions (1 mg mL-1) were prepared by application of methanol as the solvent. Out of this standard stock, different working stocks of 5, 10, 20, 50, 100, 200, 500 µg/mL aqueous standards (AQ STD 1-7) were prepared. For the IS, oxcarbamazepine (1 mg mL-1) was prepared using methanol as the solvent and intermediate stock (10 µg/mL) of this was prepared using the same method. After adding 20 µL AQ-STD-1, the plasma quality control samples were prepared with the concentrations of 0.5 µg/mL as lower limit of quantitation (LLOQ), 1.5 µg/mL as low quality control (LQC), 25 µg/mL as medium quality control (MQC) and 40 µg/mL as high quality control (HQC). Selectivity was established by injecting six samples at the LLOQ level. Through comparing the mean peak response obtained by injecting blank serum samples with mean peak response of LLOQ (0.5 µg/mL), the six blank serum samples were tested for interference. Specificity was evaluated by injecting extracted blank serum and comparing any interference with the response of the extracted LLOQ samples with IS. The results obtained were in acceptance criteria of not more than 20% interference at retention time of drug and not more than 5% at the retention time of the IS.

One hundred and eighty microliter (180 µL) blank human serum was collected from the control group and taken into the centrifuge tube. Subsequently, an accurate volume of 20 µL working stock solutions of pralidoxime was spiked and 20 µL of IS working stock solution (10 µg/mL of Oxcarbamazepine) was added to the tube and vortexed for 30 seconds. Then 300 µL of 20% TCA was added and vortexed for 5 minutes and finally centrifuged for 5 minutes at 10,000 rpm at 4°C. The clear supernatant was transferred into HPLC vial and out of that 100 µL was injected into HPLC system.

**-Storage and disposal of residual samples:** The recovery of pralidoxime from the biological matrix was calculated by comparing the detector response obtained from extracted QC samples with the true concentration of the pure authentic standard. Recovery of pralidoxime was determined for low, medium and high quality control samples. Residual samples were stored at -70°C until the
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RESULTS

(C) Lower limit of quantitation or sensitivity: LLOQ of 0.5µg/mL was selected based on the lowest concentration that was expected in the current study. Accuracy and precision was carried out and it was found to be within the acceptable limit (Figure 1).

(D) Accuracy and precision: Intraday and interday accuracy and precision was carried out for the QC samples by analysing six matched samples at each level of QC samples. The results were found to be within the acceptable criteria. Hence, the method is accurate and precise (Table 2).

Figure 1. high-performance liquid chromatograms: (A) typical chromatogram of blank human serum (B) typical standard chromatogram of pralidoxime and IS (C) representative chromatogram of pralidoxime at LLOQ

end of the study period. At the end of study, biological samples were disposed based on the disposal protocol of Manipal College of Pharmaceutical Sciences.

Measurement of erythrocyte acetylcholinesterase and creatinine clearance

Using E.I. Dupont systems (Wilmington, DE, USA), the level of AChE was measured before and pralidoxime therapy. Using crockcroft-gault formula, creatinine clearance of each patient was calculated.

Statistical analysis

Data were analyzed by application of the SPSS for windows (SPSS Inc., Chicago, IL, USA). Results are shown with mean and standard deviation (SD) for variables with normal distribution and with median and interquartile range (IQR) for variables without normal distribution. Categorical data are shown with frequency and percentage. Using the Spearman's rank correlation test, the relationship between BPL and creatinine clearance was analysed. Using paired t test, the difference of serum level of AChE in prior and after pralidoxime therapy was analysed.

RESULTS

Patients' demographic and clinical manifestations
Out of 113 OP poisoned patients during the study period, 30 patients met the inclusion criteria. They had 21 to 30 years of age and the majority of them were men (60%). All patients were presented with the classic muscarinic features of OP poisoning including miosis, dyspnea, bronchorrhea, vomiting, lacrimation and sialorrhea. Twenty-five patients were included in the study group and five patients were included into the control group. In the study group, (a) seven patients received intermittent dosing (1 g/q8h), (b) thirteen patients received 500 mg/h continuous infusion and (c) five patients received 1g/h continuous infusion of pralidoxime.

HPLC method for measurement of blood level of pralidoxime

(A) Specificity and selectivity: Blank serum was collected from 6 different human volunteers and checked for selectivity. It was extracted with and without IS to assess the specificity of the method. The pralidoxime and the IS analytes were well separated from the co-eluted components under the optimized chromatographic conditions. The mean (SD) retention time for pralidoxime was found to be 9.9 (0.4) minutes while for oxcarbamazepine was 17.4 (0.2) minutes. The total run time was 22 minutes. The peaks were in good shape and completely resolved from serum components. No interferences from the serum matrix were observed at the retention time of drug and the IS. The chromatogram revealed little interference which did not affect the drug substance and the IS. The chromatograms of blank human serum and the standard chromatogram of pralidoxime with IS are shown in figure 1.

(B) Linearity: The developed HPLC method was linear over the range of 0.5-50 µg/mL (Table 1). The correlation coefficient was found to be greater than 0.99 (Figure 2).
The method was able to deliver the consistent recovery in all of the QC levels. The recovery was found to be greater than 65% (Table 2).

The stability experiments were aimed at testing all possible conditions that the samples might experience after collecting and prior the analysis. The stability of the drug was evaluated at two QC levels (LQC and HQC), short term (24 hours after starting the treatment), freeze thaw (3 cycles) and long term (30 days after the treatment). The results showed that the pralidoxime was stable in human serum for about one month when stored in the frozen state (-70°C).

Blood level of pralidoxime versus clinical outcome

In the intermittent dosing group the serum level showed a maximum peak concentration of 34.2 (10.22) µg/mL at 30 minutes after injection of pralidoxime which dropped to a low trough level of 4.63 (5.26) µg/dL at 8th hour; just before the next dose. The median (IQR) of BPL in intermittent dosing was comparatively lower than patients treated with continuous infusion (Table 3). The highest BPL was maintained in 1 g/h group with median (IQR) serum level of 38.86 (16.75).

Blood level of pralidoxime versus clinical outcome

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Blood level of pralidoxime versus AChE level

The reactivation rate of AChE was higher in continuous infusion groups compared to intermittent dosing. This may be the explanation of less incidence of intermediate syndrome in the continuous infusion group. The reactivation of AChE was statistically significant in all groups after analysis with paired t test (Table 4). Comparison of AChE before and after pralidoxime therapy showed that higher BPL was associated with greater reactivation of AChE (Table 3 and 4).

Blood level of pralidoxime versus adverse effects

Patients with higher BPL had higher systolic blood pressure (Figure 3). In the present study, two patients suffered from severe hypotension because of high BPL. The two patients were admitted after consumption of 100 mL of Chlorpyrifos and developed severe hypertension on the second day of admission with very high systolic blood pressure of 180

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**Table 1. Pralidoxime concentration and peak area ratio**

<table>
<thead>
<tr>
<th>Pralidoxime concentration (µg/mL)</th>
<th>Peak area ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.0586</td>
</tr>
<tr>
<td>1</td>
<td>0.0976</td>
</tr>
<tr>
<td>2</td>
<td>0.1732</td>
</tr>
<tr>
<td>5</td>
<td>0.4192</td>
</tr>
<tr>
<td>10</td>
<td>0.8102</td>
</tr>
<tr>
<td>20</td>
<td>1.6406</td>
</tr>
<tr>
<td>50</td>
<td>3.6903</td>
</tr>
</tbody>
</table>

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**Table 2. Parameters of bioanalytical validation**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (µg/mL); range</td>
<td>0.5-50</td>
</tr>
<tr>
<td>Correlation coefficient (r²)</td>
<td>0.9983</td>
</tr>
<tr>
<td>Sensitivity (LLOQ); (µg/mL)</td>
<td>0.5</td>
</tr>
<tr>
<td>Recovery percentage (Average of three levels)</td>
<td>78.37 (5.13)</td>
</tr>
<tr>
<td>Precision at QC levels (% CV); mean (SD)</td>
<td></td>
</tr>
<tr>
<td>LLOQ</td>
<td>15.21 (5.34)</td>
</tr>
<tr>
<td>LQC</td>
<td>5.45 (1.56)</td>
</tr>
<tr>
<td>MQC</td>
<td>8.44 (2.63)</td>
</tr>
<tr>
<td>HQC</td>
<td>6.52 (5.15)</td>
</tr>
<tr>
<td>Accuracy at QC levels (% ); mean (SD)</td>
<td></td>
</tr>
<tr>
<td>LLOQ</td>
<td>90.15 (5.35)</td>
</tr>
<tr>
<td>LQC</td>
<td>95.54 (3.67)</td>
</tr>
<tr>
<td>MQC</td>
<td>104.92 (3.85)</td>
</tr>
<tr>
<td>HQC</td>
<td>96.14 (3.55)</td>
</tr>
</tbody>
</table>

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**Table 3. Blood level of pralidoxime in different dose regimens**

<table>
<thead>
<tr>
<th>Pralidoxime dosing</th>
<th>Blood level of pralidoxime (µg/mL); median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>Intermittent (1 g/q8h)</td>
<td>4.63 (5.26)</td>
</tr>
<tr>
<td>Trough</td>
<td>4.63 (5.26)</td>
</tr>
<tr>
<td>Peak</td>
<td>34.2 (10.22)</td>
</tr>
<tr>
<td>Continuous (500 mg/h)</td>
<td>20.76 (10.15)</td>
</tr>
<tr>
<td>Continuous (1 g/h)</td>
<td>38.86 (16.75)</td>
</tr>
</tbody>
</table>

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**Figure 2. Linearity plot for pralidoxime in human serum**

**Figure 3. Blood level of pralidoxime versus adverse effects**

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Table 4. Comparison of serum level of AChE before and after pralidoxime therapy

<table>
<thead>
<tr>
<th>Pralidoxime dosing</th>
<th>Initial AChE level (IU/L); mean (SD)</th>
<th>Final AChE level (IU/L); mean (SD)</th>
<th>Increase of AChE (IU/L); mean (SD)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermittent</td>
<td>3520.2 (2773.1)</td>
<td>4276.4 (2831.8)</td>
<td>756.2 (158.6)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>500 mg/h</td>
<td>67 (76.9)</td>
<td>3392.3 (803.9)</td>
<td>3325.3 (740.3)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>1 g/h</td>
<td>515.7 (890.4)</td>
<td>4149.1 (855.6)</td>
<td>3633.4 (823.3)</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

Figure 3. Correlation of blood level of pralidoxime and systolic blood pressure

mmHg and 190 mmHg after continuous infusion of pralidoxime on the regimens of 500 mg/h and 1 g/h; respectively. The corresponding blood levels of pralidoxime were 58.76 µg/mL and 64.84 µg/mL, respectively. Following reduction of dose of pralidoxime to 125 mg/h, blood pressure of the patients was restored.

Blood level of pralidoxime versus creatinine clearance

Median (IQR) of creatinine clearance in patients was 96.6 (20.6) and median (IQR) of trough pralidoxime serum level was 22.4 (18.0). Analysis showed a statistically significant inverse correlation between creatinine clearance and trough pralidoxime serum level (r = -0.909; P < 0.001).

DISCUSSION

In this pilot study, blood levels of pralidoxime were measured using a HPLC method. This bioanalytical method was developed and validated for the measurement of pralidoxime in human serum in the range of 0.5-50 µg/mL. The correlation coefficient was found to be greater than 0.99. Accuracy and precision was found to be within the acceptable limits. The method was able to deliver consistent recovery in all QC levels. The recovery was found to be greater than 65%.

For detection of pralidoxime in biological samples various methods including ion exchange, size-exclusion, reversed-phase, ligand-exchange chromatography and ion pair chromatography have been used (14). For this purpose, HPLC is a method which has been recently introduced (13). Houze et al. estimated blood level of pralidoxime through HPLC technique with electrochemical detection in human serum using guanosine as the IS (13). Their method was similar to ours, though we used oxcarbamazepine as the IS. Their assay was linear between 0.25 and 50 gm/L with a quantification limit of 0.2 gm/L. The analytical precision was satisfactory, with variation coefficients of lower than 10%. Their quantification limit was similar to our method (13).

Kinetic studies are important to determine the minimum effective serum concentration of pralidoxime in OP poisoned patients. In the present study, BPL was measured in OP poisoned patients treated with different dose regimens of pralidoxime. The BPL in intermittent dosing (1 g/q8h) was lower than continuous pralidoxime infusion. Moreover, the mean serum concentration of patients treated with 1 g/h continuous infusion was the highest.

To date, most of the studies have shown that the minimum BPL required to reactivate AChE is 4 µg/mL with a range of 4-12 µg/mL (6,7). In a study carried out on healthy adults, bolus intravenous 7.5–10 mg/kg pralidoxime chloride was needed to produce BPL of 4 µg/mL or greater at 1 hour after administration (7). However, kinetics of pralidoxime in healthy volunteers is entirely different from poisoned patients (8). Jovanovic in a study compared BPL in healthy volunteers with OP poisoned patients after intramuscular administration of 1g pralidoxime chloride. He showed 1.5 times greater elevation of the BPL in OP poisoned patients (8). This may be due to the probable prolongation of “effective half-life” of pralidoxime in OP poisoned patients and this prolongation may be beneficial for them (8). Jovanovic also showed that AChE reactivation following pralidoxime therapy, not only depends on the plasma concentration of the oxime but also on the plasma concentration of the OP agent. He found that oxime concentrations of about 4 µg/mL are effective provided if the plasma concentrations of OP compounds are below 30 µg/L (8). During the initial period of exposure, especially during cholinergic crisis, it is necessary to maintain a higher dose of pralidoxime which should be slowly tapered depending on the clinical symptoms. Schexnayder et al. in a study on OP poisoned children showed that pralidoxime can be maintained up to an average of 22 µg/mL after intravenous administration of a loading dose of 15-50 mg/kg of pralidoxime, followed by a continuous infusion of 10-20 mg/kg/h which is similar to our findings (11).

In this study, we found that AChE reactivation is greater in continuous infusion compared to intermittent dosing.
Similar results was found in a randomized controlled trial conducted by Eddleston et al., which showed that 500 mg/h continuous infusion of pralidoxime can significantly reactivates erythrocyte AChE in comparison to placebo (12).

In this study, we showed that high BPL can induce hemodynamic changes as two of our patients who had over 50 μg/mL BPL developed hypertension. Calesnick et al. similarly reported that intramuscular administration of 30 mg/kg pralidoxime chloride results in ECG changes with T-wave elevation and increased blood pressure. Medicis et al. also demonstrated that 80 μmol/L BPL (corresponding to 14 mg/L of pralidoxime chloride) was associated with dizziness, hypertension and blurred vision (16). BPL mainly depends on the creatinine clearance of the patient. In our study we observed that BPL inversely correlated to creatinine clearance. This could be due to the fact that pralidoxime is primarily excreted through kidneys (17). In this study, we showed that continuous infusion of pralidoxime is altogether more beneficial than intermittent dosing. Likewise, Pawar et al. in a comparative study on 200 OP poisoned patients revealed that 1 g/h continuous infusion of pralidoxime reduces morbidity and mortality, and is more effective than repeated bolus injection of 1 g every 4 hours (9).

CONCLUSION

HPLC can be used as alternative method for measurement of pralidoxime level in blood. Continuous infusion of pralidoxime maintained a steady higher blood concentration compared to intermittent dosing with vast fluctuations. The reactivation rate of AChE was higher in continuous infusion compared to intermittent dosing. Hence, continuous infusion of pralidoxime can more rapidly recover the OP poisoned patients with less morbidity.

LIMITATIONS

The sample size used in the present study was low because many patients did not have a clear history. Moreover, the level of OP agent consumed was not measured for any patient.

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