Abstract

The pralidoxime chloride (PAM) cartridge in a reusable autoinjector consists of 300 mg/mL of pralidoxime chloride in 2.2 mL solution with a shelf life of one year. The present study is aimed at evaluating the antidotal efficacy of PAM filled in the cartridges at (stored at room temperature of 25-35°C) different time points using three different methods to determine the shelf life. The study showed that administration of atropine sulfate or freshly prepared pralidoxime chloride alone did not offer appreciable protection as measured by Protection Index (PI) against either diisopropylphosphorofluoridate (DFP) or dichlorvos (DDVP) in male mice. The combined treatment of atropine sulfate and samples of pralidoxime chloride, one after the other but close in time, showed far better protection. Atropine sulfate antagonizes the actions of acetylcholine and PAM reactivates the inhibited acetylcholinesterase (AChE) enzyme. In the case of DDVP, treatment with atropine sulfate and all the samples of pralidoxime chloride (freshly prepared, and 3, 6, 12, 24 and 36 months old) offered a PI of 26.9. On the other hand, DFP intoxication and subsequent treatment with atropine sulfate and all the samples of PAM showed varying PI. It was observed that maximum PI (38.3) could be achieved with 12 months old PAM sample compared to freshly prepared solution. Minimum PI (13.3) was observed with 36 months old PAM sample. This decrease in PI can not be attributed to the less percent reactivation (91.3%) of isopropyl methyl phosphonofluoridate (Sarin) inhibited electric eel AChE by PAM, as both the parameters i.e. PI (26.9) of DDVP and estimation of active PAM (99.5±5.9%) are not in agreement. We assume that the said change may be due to biological variation and high toxicity of DFP (4 times more toxic than that of DDVP). It may be concluded that (i) Pralidoxime chloride filled and stored in autoinjector cartridges has storage stability of more than 2 years, (ii) it also has an appreciable activity in terms of protection index, percent reactivation of sarin inhibited electric eel acetylcholinesterase and (iii) estimation of active PAM for three years.

Keywords

Diisopropylphosphorofluoridate, Dichlorvos, Pralidoxime chloride, Atropine sulfate, Autoinjector

Introduction

Organophosphorus (OP) poisons inhibit the enzymatic hydrolysis acetylcholine (ACh) by acetylcholinesterase (AChE). This leads to the accumulation of ACh at nerve endings resulting in prolonged and intensified actions on the effector site (1, 2). The nerve agents (also known as nerve gases) are OP compounds. All OP compounds do not qualify as war gases and are not a threat, due to their differential toxicity.
Some of the less toxic OP compounds are also used as insecticides (1-3). The nerve agents are tabun, sarin, soman and Vx, and they are classified under Schedule I of Chemical Weapons Convention (CWC) (4-6). The absorption of these OP compounds into the system is through inhalation, dermal absorption, and through mucous membranes (7).

The nerve agents are also a potential threat from the terrorist organizations (8). During the World War II huge quantities of the nerve agents were stockpiled, and there were allegations the same being used during Iraq-Iran war (9, 10). In 1995, the Aum Shinrikyo group of Japan used extremely toxic nerve agent, sarin in a Tokyo subway killing 12 people and injuring about 5000 people (11). Though the CWC has been signed and ratified by more than 180 countries, the threat persists, and several countries have developed preparedness for such an eventuality (3). Further, accidental, suicidal and homicidal OP poisoning are common leading to the possible loss of life (12).

As soon as organophosphorous poisons enter the system, symptoms of poisoning appear and affect the muscarinic and nicotinic receptors. These effects include constriction of pupil (miosis), increased production of saliva, running nose, increased perspiration, urination, defecation, bronchosecretion, bronchoconstriction, decreased heart rate and blood pressure, muscular twitches and cramps, cardiac arrhythmias, tremors and convulsions. The most critical effects are paralysis of respiratory muscles and inhibition of respiratory centre. Ultimately death results due to the respiratory arrest (13). If the concentration of the agent is high, death is immediate. The treatment of nerve agent casualty requires artificial respiration and drug treatment. The recommended drugs are atropine sulfate and pralidoxime chloride (14). Atropine sulfate is a competitive inhibitor of muscarinic receptors and recommended human dose at the first instance is 2 mg intramuscularly and subsequently increased to 4 to 6 mg, and some times higher. Pralidoxime chloride is used as a cholinesterase reactivator and recommended dose is 500 to 1000 mg intramuscularly or intravenously (15). Death following nerve agent exposure is very rapid and in the field it is not possible for medical personnel to administer the drugs when large numbers of persons are affected. Further, a majority of deaths due to OP poisoning may be avoided by using the antidotes well in time. Due to practical problems i.e. quick administration (intramuscular or intravenous) of the antidotes at the site of incidence is difficult in the absence of trained personnel (16). To address this practical drawback autoinjectors are being developed by several countries for the immediate field administration of the drugs by the affected individual himself or by his buddy (17). These Autoinjectors commonly contain two types one for atropine sulfate and the other for pralidoxime chloride together but historically they were used one after the other (3). Atropine sulfate in solution is a standard preparation and routinely used as a preanaesthetic medication. Pralidoxime chloride solution is uncommon and of varying stability at different storage conditions.

Keeping all the above points in view, reusable autoinjectors have been developed, in which once the drugs shelf life expires, the drug cartridge can be replaced with fresh cartridges (3). The pralidoxime chloride cartridge consists of 300 mg/mL of pralidoxime chloride in 2.2 mL solution with a labeled shelf life of one year. The present study is aimed to evaluate the antidotal efficacy of pralidoxime chloride solution filled in the cartridges at different time points using three different methods to ascertain its actual shelf life.

Materials and Methods

Animals: Randomly bred Swiss albino male mice weighing between 30-35 g from the Institute’s animal facility were used for the study.
They were housed in polypropylene cages (five mice per cage) under controlled environmental conditions with free access to pellet diet (Ashirwad Brand, India) and water. The care and maintenance of animals were as per the approved guidelines of the ‘Committee for the Purpose of Control and Supervision of Experiments on Animals’ (CPCSEA, India). This study had the approval of the Institute’s Animal Ethical Committee.

Chemicals

Diisopropylphosphorofluoridate (DFP) and isopropyl methyl phosphono-fluoridate (Sarin) were synthesized in the Process Technology Development Division and was found to be more than 99% pure by gas chromatographic analysis. Dichlorvos or DDVP (2,2-Dichloroethanol dimethyl phosphate) and other analytical grade chemicals were purchased from the trade. Extreme care was taken during the synthesis and storage of DFP and sarin, as per approved guidelines of the institute.

Autoinjectors and Pralidoxime Cartridges: The technology of production of the autoinjectors and the drug cartridges has been transferred to the industries by Defence R & D Organisation (3), and the same pralidoxime cartridges have been used in this study. These autoinjectors are reusable, i.e. once the drug shelf life expires, the cartridges can be replaced with fresh cartridges. All the cartridges were stored at room temperature (25-35°C).

Drug assay

The concentration of pralidoxime chloride was determined spectrophotometrically as per the Indian Pharmacopeia method (18). Briefly, to the diluted solution of pralidoxime chloride, 1 N sodium hydroxide was added and OD was measured at 336 nm in a Spectronic 1201 spectrophotometer (Spectronic Corporation, USA) and compared with a standard.

Percent reactivation of sarin (isopropyl methyliophosphonofluoridate) inhibited Electric eel acetylcholinesterase (AChE) by pralidoxime chloride: To measure the AChE, the following protocol was adopted. Five units of Electric eel AChE (Sigma Cat. No. C-2888) were taken in 1.0 ml phosphate buffer (0.1 M; pH 7.6). This was incubated for 10 minutes with 2.0-8.0 x 10⁻⁸ M sarin to obtain an inhibition of the enzyme activity in the range of 95-98%. Two 0.2 ml aliquots from this inhibition cocktail were taken in two glass tubes. One was incubated for 10 minutes with 2 µl of 0.176 M or 3.52 x 10⁻⁴ M pralidoxime chloride prepared in distilled water. Another tube received equivalent quantity of distilled water. To determine control enzyme activity, similar preparations were made without the addition of sarin. AChE activities were determined using the method of Ellman et al. (1961) as described by Steck and Kant (1974) (19). In a cuvette, 5-10 µl enzyme containing samples were taken and 3 ml of Phosphate Buffer (0.1 M; pH 8.0) was added to it. Thereafter, 100 µl 5 mM 5,5'-dithiobis 2-nitro benzoic acid (DTNB) in phosphate buffer (0.1 M; pH 7.0) was added and incubated at 37°C for two minutes. The enzyme reaction was started by the addition of 50 µl of 75 mM acetylthiocholine iodide as substrate. The enzyme activity was determined by recording the change in absorbance per minute for 4 minutes at 410 nm in Specord-200 UV-Vis spectrophotometer. Percent reactivation was calculated considering control enzyme activity as 100% in presence of pralidoxime chloride.

Determination of median lethal dose (LD₅₀) and Protection Index (PI): The LD₅₀ values of DFP and DDVP dissolved in distilled water, were determined in male mice following subcutaneous (s.c.) route of administration. The observation period for mortality of the treated animals was 24 hours. The dilution of the compounds was done so that the injectable volume was approximately 0.2 ml in all the experiments. The LD₅₀ determinations were done by Dixon’s Up
and Down method (20) for DFP exposed animals, and by the moving average method of Gad and Weil (21) using two to four groups, each group consisting of four animals. Protection Index (PI) was determined following the formula given below:

\[
\text{PI} = \frac{\text{LD}_{50} \text{ of DFP or DDVP in Atropine + Pralidoxime chloride treated animals}}{\text{LD}_{50} \text{ of DFP or DDVP in distilled water treated animals}}
\]

Where, the treatment dose of atropine sulfate and pralidoxime chloride was 10 mg/kg (i.p.) and 30 ml/kg (i.p.) respectively. Freshly prepared solution of atropine sulfate and freshly diluted solution of pralidoxime chloride were used in the study. For dilution of pralidoxime chloride, required volume was taken from the autoinjector cartridge using sterilized needle and syringe and diluted in distilled water. Both the drugs i.e. atropine and pralidoxime chloride were administered immediately after injection of either DFP or DDVP.

**Table 1:** Median lethal dose (LD_{50}) of Diisopropylphosphorofluoridate (DFP) and Protection Index (PI) obtained by the treatment with Atropine sulfate (10 mg/kg) and/ or Pralidoxime chloride (PAM; 30 mg/kg) in male mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>LD_{50} (mg/kg)</th>
<th>Protection Index (PI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFP alone</td>
<td>2.9 (1.85-4.59)</td>
<td>1</td>
</tr>
<tr>
<td>DFP + Atropine</td>
<td>6.6 (4.17-10.33)</td>
<td>2.2</td>
</tr>
<tr>
<td>DFP + PAM Freshly prepared</td>
<td>4.9 (3.26-7.46)</td>
<td>1.7</td>
</tr>
<tr>
<td>DFP + Atropine + PAM Freshly prepared</td>
<td>97.7 (64.63-147.6)</td>
<td>33.5</td>
</tr>
<tr>
<td>DFP + Atropine + PAM, 3 mo. old^{a}</td>
<td>97.7 (64.63-147.6)</td>
<td>33.5</td>
</tr>
<tr>
<td>DFP + Atropine + PAM, 6 mo. old^{a}</td>
<td>77.8 (46.52-130.1)</td>
<td>26.7</td>
</tr>
<tr>
<td>DFP + Atropine + PAM, 12 mo. old^{a}</td>
<td>111.7 (66.81-186.9)</td>
<td>38.3</td>
</tr>
<tr>
<td>DFP + Atropine + PAM, 24 mo. old^{a}</td>
<td>56.0 (33.48-93.69)</td>
<td>19.2</td>
</tr>
<tr>
<td>DFP + Atropine + PAM, 36 mo. old^{a}</td>
<td>39.0 (23.31-65.24)</td>
<td>13.4</td>
</tr>
</tbody>
</table>

^{a} Samples taken from the cartridges of Autoinjector. The LD_{50} determinations was done by Dixon’s Up and Down method (20). All atropine solutions were freshly prepared.

Statistical analysis: Results are expressed as mean±SEM. Data were analyzed by Student’s t-test. Statistical significance was considered at p<0.05.

**Results and Discussion**

Table 1 depicts median lethal dose (LD_{50}) of Diisopropylphosphorofluoridate (DFP) and Protection Index (PI) obtained by the treatment with atropine sulfate (10 mg/kg) and/ or Pralidoxime chloride (30 mg/kg) in male mice. Treatments with atropine sulfate and pralidoxime chloride individually after DFP poisoning offered a PI of 2.2 and 1.7 respectively. Treatment with combination of both the drugs i.e. atropine sulfate and pralidoxime chloride offered PI of 33.5, which was higher than the additive effects of individual drugs. Six months old pralidoxime chloride showed PI of 26.7 while, 12 month old pralidoxime chloride showed more pronounced PI (38.3). However, 24 and 36 months old pralidoxime chloride could offer less PI (19.2 and 13.4 respectively) compared to 3, 6 and 12 months old pralidoxime chloride.

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Table 2 shows median lethal dose (LD<sub>50</sub>) of DDVP and PI obtained by the treatment with Atropine sulfate (10 mg/kg) and/or Pralidoxime chloride (30 mg/kg) in male mice. Treatments with atropine alone and pralidoxime chloride alone after DDVP poisoning offered PI of 3.4 and 2.8 respectively. Treatment with combined administration of the two drugs (atropine sulfate and pralidoxime chloride) offered PI of 26.9, which was significantly more pronounced than the additive effects of individual drugs. Freshly prepared solution of pralidoxime chloride, and 3, 6, 12, 24 and 36 months old showed PI of 26.9. On the other hand freshly prepared solution of pralidoxime chloride at a dose of 15 mg/kg (50% of the actual dose) could offer less PI (13.4) compared to all other PAM samples.

Table 2: Median lethal dose (LD<sub>50</sub>) of Dichlorvos (DDVP) and Protection Index (PI) obtained by the treatment with Atropine sulfate (10 mg/kg) and/or Pralidoxime chloride (PAM; 30 mg/kg) in male mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; (mg/kg)</th>
<th>Protection Index (PI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDVP alone</td>
<td>11.9 (7.78-18.17)</td>
<td>1</td>
</tr>
<tr>
<td>DDVP + Atropine</td>
<td>40.0 (0.00-0.00)</td>
<td>3.4</td>
</tr>
<tr>
<td>DDVP + PAM Freshly prepared</td>
<td>33.6 (22.01-51.39)</td>
<td>2.8</td>
</tr>
<tr>
<td>DDVP + Atropine + PAM Freshly prepared</td>
<td>320.0 (25.06-4084)</td>
<td>26.9</td>
</tr>
<tr>
<td>DDVP + Atropine + PAM, 3 mo. old&lt;sup&gt;a&lt;/sup&gt;</td>
<td>320.0 (89.56-1143)</td>
<td>26.9</td>
</tr>
<tr>
<td>DDVP + Atropine + PAM, 6 mo. old&lt;sup&gt;a&lt;/sup&gt;</td>
<td>320.0 (89.56-1143)</td>
<td>26.9</td>
</tr>
<tr>
<td>DDVP + Atropine + PAM, 12 mo. old&lt;sup&gt;a&lt;/sup&gt;</td>
<td>320.0 (25.06-4084)</td>
<td>26.9</td>
</tr>
<tr>
<td>DDVP + Atropine + PAM, 24 mo. old&lt;sup&gt;a&lt;/sup&gt;</td>
<td>320.0 (25.06-4084)</td>
<td>26.9</td>
</tr>
<tr>
<td>DDVP + Atropine + PAM, 36 mo. old&lt;sup&gt;a&lt;/sup&gt;</td>
<td>320.0 (89.56-1143)</td>
<td>26.9</td>
</tr>
<tr>
<td>DDVP + Atropine + PAM (15 mg/kg)</td>
<td>160.0 (33.36-767.6)</td>
<td>13.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Samples taken from the cartridges of Autoinjector. The LD<sub>50</sub> determinations was done by the moving average method of Gad and Weil using two to four groups, each group consisting of four animals (21). All atropine solutions were freshly prepared.
There is an advantage to the reusable autoinjectors over the single time autoinjectors, since the drug cartridges can be changed after the expiry of the drugs. The autoinjectors are very easy to use in emergency. They are preferred over manual injection of the drugs, since the drug is delivered in the various layers of muscle unlike the conventional (manual) injection where the whole drug is released at one place (3). The absorption of the drug is expected to be faster in the autoinjector delivery (22).

For the treatment of poisoning of the various chemical warfare agents, specific antidotes are available for the nerve agents. An antagonist of acetylcholine, such as atropine sulfate and a reactivator of inhibited AChE such as pralidoxime chloride are the recommended drugs for the treatment of organophosphorous intoxications, including the nerve agents (23-25). The role of atropine sulfate is as a competitive inhibitor of ACh at the muscarinic receptors. The inhibition of cholinesterase by the nerve agents stimulates not only the muscarinic receptors but also the nicotinic receptors (26). The phosphorylated AChE reacts very slowly or not at all with water. However, if more reactive OH groups in the form of oximes are provided, reactivation occurs more than a million times faster (12). Hence for treating the nerve agent poisoning along with atropine sulfate, an AChE reactivator is also required such as the pralidoxime chloride, to counter the nicotinic effects. We observed based on Protection Index (PI), the administration of atropine sulfate and pralidoxime chloride together resulted better protection compared to the individual protection from each drug. It has also been observed in previous study that the protection of nerve agent poisoning by the combined administration of cholinolytic and cholinesterase reactivator is better than the individual drugs (27, 28).

As a result of sarin attack on the Tokyo subway, various threat analyses have cautioned that terrorists may try to use chemical warfare agents again. In such a situation the autoinjectors will be very useful for the medical team. The autoinjectors are also useful in the case of OP insecticide poisoning, as they can be stored in

Table 3: Percent reactivation of Isopropyl methyl phosphonofluoridate (Sarin) inhibited Electric Eel Acetylcholinesterase (AChE) by Pralidoxime chloride (PAM), and Estimation of active PAM

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Reactivation of Sarin inhibited Electric Eel AChE By PAM</th>
<th>% of active PAM (Photometric assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAM, 3 mo. old</td>
<td>118.9</td>
<td>100.0 ± 2.3</td>
</tr>
<tr>
<td>PAM, 6 mo. old</td>
<td>116.9</td>
<td>102.0 ± 3.1</td>
</tr>
<tr>
<td>PAM, 12 mo. old</td>
<td>95.2</td>
<td>106.0 ± 3.2</td>
</tr>
<tr>
<td>PAM, 24 mo. old</td>
<td>96.0</td>
<td>98.4 ± 5.1</td>
</tr>
<tr>
<td>PAM, 36 mo. old</td>
<td>91.3</td>
<td>99.5 ± 5.9</td>
</tr>
</tbody>
</table>

For % of active PAM the values are expressed as mean±SEM, n=3. p<0.05 compared to PAM, 3 months old.

a Samples taken from the cartridges of Autoinjector.
health centers. However, their effectiveness for different OP pesticides varies (3).

Inhalation and dermal absorption are the major routes of entry of the OP insecticides including nerve agents. As weapons, nerve agents are dispersed as aerosols and produce vapours under normal atmospheric conditions. For the experimental work on antidote evaluation, the OP compounds are either administered subcutaneously or by inhalation (3). Keeping this point in view, in the present study both the OP compounds i.e. DFP and DDVP were administered subcutaneously. However, sarin was used in vitro for percent reactivation of sarin inhibited electric eel AChE by pralidoxime chloride. In addition, active pralidoxime chloride was also estimated following spectrophotometric method (18).

The present study also showed that administration of atropine sulfate or freshly prepared pralidoxime chloride individually did not offer appreciable protection as measured by PI against either DFP or DDVP in male mice. The combined treatment of atropine sulfate and all the samples of pralidoxime chloride, one after the other but close in time, showed far better protection. Atropine sulfate antagonizes the actions of ACh and pralidoxime chloride reactivates the inhibited AChE enzyme (12). In the case of DDVP, treatment with atropine sulfate and all the samples of pralidoxime chloride (freshly prepared, and 3, 6, 12, 24 and 36 months old) offered a PI of 26.9. Contribution of pralidoxime chloride towards the protection was also confirmed by administering it in its 50% dose (15 mg/kg), which could offer PI of 13.4 (about 50% less). On the other hand, poisoning with DFP and subsequent treatment by atropine sulfate and all the samples of pralidoxime chloride showed varying PI, and it was found to be maximum (PI 38.3) with 12 months older sample of pralidoxime chloride which was more than the freshly prepared solution. Further, minimum PI (13.4) was observed with 36 months old sample of pralidoxime chloride. We can not attribute this depletion in PI to the value of percent reactivation (91.3%) of sarin inhibited electric eel AChE by pralidoxime chloride. Because both the parameters i.e. PI (26.9) of DDVP and estimation of active pralidoxime chloride (99.5±5.9%) are not in agreement. At this juncture it may be stated that due to biological variation and high toxicity of DFP (4 times more toxic than that of DDVP) the noted variation has taken place.

Pralidoxime chloride solution is not a routinely used medication. A reusable autoinjector is necessary, so that drug cartridges can be stored. Even if they are expired, it is still recommended to store the drugs in case of nerve agent terrorist attack as huge quantities of cartridges will be required which will take a long time to manufacture. This study shows even if the pralidoxime chloride solution is stored at room temperature it is stable for 3 years.

Conclusion
(i) Pralidoxime chloride solution filled and stored in autoinjector cartridges has shown appreciable activity in terms of protection index, percent reactivation of sarin inhibited electric eel acetylcholinesterase and estimation of active pralidoxim chloride for three years. (ii) The results also suggest that storage stability of pralidoxime chloride filled in autoinjector cartridges is more than two years.

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