WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.990
Volume 4, Issue 6, 947-956. Research Article ISSN 2277– 7105

REACTIVATION EFFICACY OF SARIN-INHIBITED ACETYLCHOLINESTERASE BY OBIDOXIME CHLORIDE IN THE PRESENCE OF SELECTED EXCIPIENT AND SEPTUM MATERIALS AT DIFFERENT TEMPERATURE

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Article Received on
28 March 2015,

Revised on 21 April 2015,
Accepted on 15 May 2015

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ABSTRACT

Objective The aim of this study is to propose in-vitro AChE reactivation study as a tool to check the compatibility of combinations of obidoxime chloride with different formulation excipients. **Materials and methods** Obidoxime chloride is highly water soluble drug. In this study, with the help of different types of preservatives, stabilizers and packaging materials different types of formulation combinations were developed to overcome the problem of instability. Developed different combinations of obidoxime chloride for the immediate shelf-administration were subjected for in-vitro reactivation against the inhibition of acetylcholinesterase (AChE) activities induced by sarin.

Results In-vitro reactivation studies suggest that compatibility of obidoxime chloride in autoinjector cartridge is good with bromobutyl back septum.

KEYWORDS: obidoxime, acetylcholinesterase, autoinjector and bromobutyl.

1. INTRODUCTION

Obidoxime chloride generally used to reverse the AChE inhibition caused by organophosphorous compound poisoning. Organophosphorous compounds (OP) are used widely as pesticides and nerve agents. The main use of nerve agents are as chemical warfare agent during war or as threatening agents by terrorist groups. The most commonly used nerve agents are tabun, sarin, soman and VX. Nerve agents inhibit acetylcholinesterase (AChE) by irreversibly binding to the catalytic site of the enzyme (Taylor et al., 1995). This resulting in

acute toxicity and may lead to apnea, respiratory paralysis and ultimately death (Worek et al., 2002;Cannard K 2006;Weiner et al.,2007).

The standard treatment of nerve agent poisoning includes a muscarinic antagonist e.g. Atropine sulphate and a reactivator of inhibited AChE by an oxime. Currently recommended oximes are Pralidoxime chloride (2-PAM; 2[{hydroxyiminomethyl]-1-methylpyridinium chloride) and Obidoxime chloride [{(1, 1'-oxybis-methylene) bis (4-hydroxyimino) methyl} pyridinium dichloride]. (Vijayaraghavan et al., 2007; Soukup et al., 2010).

A reusable autoinjector has been designed for combination of obidoxime chloride in which once the drug's shelf life expires, the drug cartridge can be replaced with fresh cartridges. Also the same autoinjector device can be reused several times with fresh cartridge, in case of mass casualty management as the treatment of OP poisoning must begin within minutes and the antidotes have to be administered with the autoinjectors by deep intramuscular route (Konidaris et al., 2013; Soukup et al., 2012).

In the current study different formulation combinations of obidoxime chloride developed with different preservatives and backseptum materials at different temperature to check the compatibility of obidoxime chloride. In-vitro reactivation of AChE is the main action of obidoxime chloride. Here in-vitro reactivation of AChE is proposed as the method to check the compatibility of different formulation excipients. All the formulation combinations developed were tested as reactivators of sarin inhibited electric eel AChE in vitro. The chemical evaluation of formulations was also done by literature methods (Radha Sharma et al., 2009; Radha Sharma et al., 2010; Radha Sharma et al., 2010).

Packaging is a major source of particulate contamination and can contribute to physical and chemical degradation of the product. Rubber formulations are used as rubber closures (vials, cartridges); rubber plungers (syringes, cartridges); and other applications (rubber septum in dual chamber vials, rubber septum for needle introduction in administration set tubing). The most common rubber polymers used in parenteral formulations closures are natural and butyl rubber. Silicone and neoprene also are used frequently in sterile products.

The shelf life of pharmaceutical formulations can be established on the basis of kinetic and predictive studies. A pharmaceutical product may be defined as stable if it's capable of a particular formulation, in a specific container/closure system, to remain within its physical,

chemical, microbiological, therapeutic and toxicological specifications. In the way to find an effective formulation, different formulation combinations of Obidoxime chloride with different additives and packaging materials for the immediate shelf-administration were developed.

2. MATERIALS AND METHODS

2.1 Chemicals

Sarin (isopropyl methyl phosphono-fluoridate) was synthesised in the Synthetic Chemistry Division, DRDE, Gwalior and was found to be more than 99% pure by gas chromatographic analysis. Extreme care was taken during the synthesis and storage of sarin, as per approved guidelines of the institute. Atropine sulphate (>99% pure) was purchased from Merck (Germany). Obidoxime chloride was also synthesized in the Synthetic Chemistry Division DRDE Gwalior according to the reported method and its purity was checked by TLC and was found to be more than 99% pure. Electric eel AChE (EC.3.1.17), 5, 5'-dithiobis 2-nitro benzoic acid (DTNB), acetyl thiocholine iodide (ATCh), methyl-4-hydroxy benzoate (99%) and benzyl alcohol were purchased from Sigma-Aldrich (USA). General chemicals were of pharmaceutical or analytical grade obtained from Merck (India). For autoinjector cartridge materials viz., borosilicate glass cartridge (Borosilicate, India), bromobutyl septums (Bharat Rubber, India), and stainlesssteel needles (Iscon Engineering, India) were used.

2.2 Formulation development

Glass cartridge, convoluted needle and the septums were sterilized in a steam sterilizer. The needle was introduced in the glass cartridge and the back septum was placed and aligned. The Obidoxime chloride (110 mg/ml) along with atropine sulphate (1 mg/ml) cartridges were prepared with different exicipients (phenol 2% w/v, methyl paraben 0.1% w/v, benzyl alcohol 0.2% w/v) and filtered through a 0.22 -µm membrane filters (Millipore Corp., Mass, U SA). The final pH of drug solution was 4.0-4.5 and 2.2 ml of drug solution was filled in the glass cartridges, covered with the front septum under nitrogen purging and sealed with an aluminium cap using the hand operated crimping device. Different sets of autoinjector containing cartridges were used for the study and kept in a photostability chamber (Thermolabs Scientific, India) with 65 % (± 5 %) relative humidity as per the international conference of harmonization (ICH) guidelines (ICH guideline). The cartridges were stored and then checked for in-vitro reactivation potency for obidoxime chloride (Table A).

Table A: Different developed formulation combinations with their storage conditions

Backseptum used	Dose of Atropine sulphate	Dose of Obidoxime chloride in cartridge	Temperature stored at (^{o}C)	Preservative used
Neoprene	1mg/ml	110mg/ml	30,40,50	MP
	1mg/ml	110mg/ml	30,40,50	BA
	1mg/ml	110mg/ml	30,40,50	MP+P
	1mg/ml	110mg/ml	30,40,50	BA+P
Silicon	1mg/ml	110mg/ml	30,40,50	MP
	1mg/ml	110mg/ml	30,40,50	BA
	1mg/ml	110mg/ml	30,40,50	MP+P
	1mg/ml	110mg/ml	30,40,50	BA+P
Bromobutyl	1mg/ml	110mg/ml	30,40,50	MP
	1mg/ml	110mg/ml	30,40,50	BA
	1mg/ml	110mg/ml	30,40,50	MP+P
	1mg/ml	110mg/ml	30,40,50	BA+P

Where MP: Methyl Paraben; BA: Benzyl Alcohol: P: Phenol.

2.3 Studies in vitro

2.3.1 AChE activity measurement

The AChE was used from electric eel. All experiments were done in 0.1M phosphate buffer, pH 7.4, at 25°C and the substrate was acetylthiocholine (ATCh). The enzyme activity was measured spectrophotometrically according to the Ellman procedure (Ellman et al., 1961) reagent DTNB. The increase in absorbance was read at 410 nm up to 60 min. The enzyme activity was measured by change in absorbance per minute at 410 nm in Specord-200 UV-Vis spectrophotometer. Percent reactivation was calculated considering control enzyme activity as 100% in presence of obidoxime chloride.

2.3.2 Reactivation of sarin inhibited electric eel acetylcholinesterase (AChE) by different developed formulation combinations

The in vitro reactivation of sarin inhibited AChE using test oximes were carried out in triplicate in phosphate buffer (0.1 M, pH 7.4 at 37 °C) using the method of Ellman (Ellman et al. 1961). Values depicted in Tables are average of triplicate runs with relative standard deviation of ±2%. AChE stock solution was prepared in phosphate buffer pH 7.6 (0.1 M), diluted 100 times with phosphate buffer. Percentage reactivation was calculated with the following equation, % reactivation= $(E_r\text{-}E_i / E_0\text{-}E_i) \times 100$

Where E0 is the control enzyme activity at 0 min, Ei is the inhibited enzyme activity and Er is the activity of reactivated enzyme after incubation with the oxime test compounds.

Spontaneous reactivation of inhibited AChE was assayed using same protocol, the reaction mixture contained enzyme and sarin but no oxime. Under these conditions spontaneous reactivation was found to be insignificant. All the values are corrected for their oxime induced hydrolysis.

3. RESULTS

A comparative between reactivation rates of sarin inhibited electric eel AchE for all developed formulations of obidoxime chloride (11% w/v) with bromobutyl, silicone and neoprene rubber backseptums were checked by keeping the samples at different temperature 30^{o}C, 40^{o}C and 50^{o}C. The results were summarized in Table B. The enzyme activities were calculated as described by Steck and Kant 1974. Data are given as mean ± standard error. Sarin inhibited electric eel AchE enzyme by more than 98%.

In the combination containing methyl paraben and benzyl alcohol with bromobutyl backseptum the average reactivation of AChE by obidoxime chloride was more than 100 % at 30°C temperature but with silicon and neoprene backseptums it become less (>100%). In the combinations containing methyl paraben stored at 40°C the average reactivation achieved were found to be 97 % with bromobutyl backseptum and around 95% with silicon and neoprene backseptums and these values become 95% and less than 94 % in case the benzyl alcohol containing combinations (Table B). Similar pattern was noticed with combinations stored at 50°C. The average reactivation in combination of methyl paraben with bromobutyl backseptums was found to be 96% while with others it becomes decreased (95% with silicon and 94% with neoprene).

In the combinations containing phenol with methyl paraben and phenol with benzyl alohol there was a significant degradation was noticed at all the storage temperatures (< 70%). In case of combinations with bromobutyl backseptums at all storage temperature the reactivation potency was found to be between 62-76%. While in case of silicon and neoprene backseptums these values becomes 63-71%. There was no significant difference between the combination containing methyl paraben and benzyl alcohol alone with various septum materials (Table.B) at all the temperature studied.

Table 2- Reactivation efficacy of sarin-inhibited acetylcholinesterase by obidoxime chloride in the presence of selected excipient and septum materials after 1 month of storage at different temperature

Preservatives	Silicon backseptum			Bromobutyl backseptum			Neoprene backseptum		
	30°C	40°C	50°C	30°C	40°C	50°C	30°C	40°C	50°C
Methyl paraben	96.4±0.24	95.4±0.1	95.1±0.05	100.9±0.4	97.0±0.17	96.4±0.2	97.6±0.05	95.3±0.1	94.2±0.1
Benzyl alcohol	95.8±0.4	94.7±0.02	94.5±0.02	101.9±0.28	95.5±0.1	95.6±0.05	93.3±0.05	92.7±0.1	91.3±0.2
Methyl paraben + Phenol	67.7±0.05[a]	66.8±0.05[a]	67.7±0.05[a]	63.2±0.17[a]	62.7±0.2[a]	64.0±0.05[a]	65.2±0.1[a]	64.6±0.05[a]	65.8±0.05[a]
Benzyl alcohol + Phenol	71.0±0.04[a]	69.1±0.05[a]	68.5±0.05[a]	76.9±0.05[a]	67.0±0.05[a]	68.4±0.05[a]	65.3±0.1[a]	64.4±0.1[a]	63.8±0.02[a]

***** For % reactivation of AChE activity values are expressed as Mean ± SEM, n=3;

Where MP= Methyl Paraben; BA=Benzyl Alcohol; P=Phenol

a Statistically significant difference from methyl paraben.

Data are given as mean ± standard error.

4. DISCUSSIONS

Several mono-pyridinium and bis-pyridinium oximes have been synthesized and tested (Wilson and Ginsburg 1955). In the present study, the potential of reactivation of newly developed formulation combinations of obidoxime against sarin inhibited electric eel AChE tested and compared. It is well known that the inhibition of AChE activities in an organism is due the effect of the active metabolites (oxons). In vitro AChE reactivation inhibited with the sarin and other OP compounds is well documented and accepted as evaluation of oxime. (Worek et al., 2002; Uday, K. Killi.et al., 2014).

AChE, may show different inhibition, reactivation and aging kinetics (Eyer, 2003). Literature shows a less data regarding structure activity relationships for oxime efficacy (Taylor et al., 1955). The reactivation potency of obidoxime has a complex dependency on the nucleophilicity and orientation of the obidoxime as well as on the structure of the OP–AChE conjugate (Ashani et al., 1995). The mechanism by which the obidoxime exerts AChE reactivation property is based on the chemical principle that oxime reactivation occurs by the nucleophilic attack of oximate anions on the OP–AChE conjugates.

In this study, we tested different combinations of obidoximes which have only one excipient, like methyl paraben and benzyl alcohol with others having more than one excipients like methyl paraben with phenol and benzyl alcohol with phenol. In the enzyme reactivation the number of aldoxime groups is not of important. There are so many factors affect the

reactivation activity of obidoxime. Some other structural features like the number of pyridinium rings, the position of the oxime group in the pyridinium ring and the number of methylene groups in linking chain between two quaternary pyridinium rings in the molecule of reactivators that can play an important role in the reactivating efficacy of oximes (Worek et al., 2002).

Though chemical weapon may not be used in war henceforth, but terrorist organizations may use it as was chosen by the use of sarin gas in Tokyo subway (MacIIwain 1993; Morita et al., 1995; Nagao et al., 1997). Therefore preparedness for mass casualty management has to be kept ready always. The preferred antidote for nerve gas is atropine sulphate and an oxime like pralidoxime chloride or obidoxime chloride. They have to be administered by autoinjector in field conditions. So far, currently available autoinjectors consists of atropine sulphate and pralidoxime chloride or obidoxime chloride in separate autoinjectors and are advised to be administered one after other. Both atropine sulphate and the oxime are required for nerve gas management (Spohrer 1994). If it is administered as single it will take more time and more painful. Further, it has been decided to combine both atropine sulphate and pralidoxime chloride but it would be more concentrated hence atropine sulphate and obidoxime chloride combination is prepared. Obidoxime chloride is less toxic as well as less concentration is required for AChE reactivation. The recommended concentration of atropine sulphate, pralidoxime chloride and obidoxime chloride is 1 mg/ml, 300 mg/ml and 110 mg/ml (11%; 0.306 M) respectively.

In this study, materials used in making cartridges are viz bromobutyl or silicon back septum, needle, borosilicate glass cartridge, silicon oil for polishing of backseptums and also the excipients like methyl paraben or benzyl alcohol as stabilizers. The reactivation of sarin-inhibited AChE by obidoxime chloride with methyl paraben and benzyl alcohol showed a rapid increase in enzyme activity. The back septums can have an influence on the reactivation activity and stability of the product packaged in the cartridge; since it is in contact with the product .The proper functioning of the autoinjector depends on the type of back septum (Johnson et al., 2000).

In the present study atropine sulphate and obidoxime chloride combination was used with methyl paraben or benzyl alcohol as an excipient, also in the presence of bromobutyl or neoprene or silicon backseptum material. This combination is stable for 2 years at room temperature, since at 50^{o}C it is stable for one month by extrapolation method (ICH

guidelines). On the basis of this study it can be concluded that methyl paraben or benzyl alcohol can be used as preservative in obidoxime chloride combination with any kind of back septum but preferably silicon or bromobutyl.

From the results obtained it is cleared that obidoxime able to reactivate sarin-inhibited AChE with methyl paraben and benzyl alcohol with the combination of bromobutyl back septum. Obidoxime chloride with benzyl alcohol also showed good reactivation but as overall methyl paraben was superior. The combination of phenol with methyl paraben and phenol with benzyl alcohol showed poor reactivation potency. Obidoxime chloride sample with different combinations stored at 30°C temperature seems to be more potent as compare to 40°C, 50°C.

5. SUMMARY

In summary, the reactivation potency of different formulation combinations of obidoxime chloride and atropine sulphate was evaluated to design a suitable parenteral formulation. Because of its good aqueous solubility, the formulation was prepared in a totally aqueous system. Inclusion of different preservatives like methyl paraben and benzyl alcohol helped in minimizing hydrolytic degradation. Manufacturing issues such as compatibility with excipients, backseptums, closures and storage conditions were also addressed.

Although a slight degradation was seen with methyl paraben and benzyl alcohol when combined with phenol at higher temperatures (50°C). The combined treatment of atropine sulphate and obidoxime chloride showed a better protection against OP poisoning. Obidoxime chloride in autoinjector cartridge with bromobutyl back septum has shown good reactivity in terms of % enzyme reactivation of sarin-inhibited electric eel AChE. Results of in-vitro reactivation studies also suggest that storage stability of obidoxime chloride in autoinjector cartridge is good with bromobutyl back septum. This also propsed in-vitro reactivation studies as a tool to check the compatibility between API (active pharmaceutical ingredients) with different formulation excipients in the development of new formulations.

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