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# The estimation of oxime efficiency is affected by the experimental design of phosphylated acetylcholinesterase reactivation

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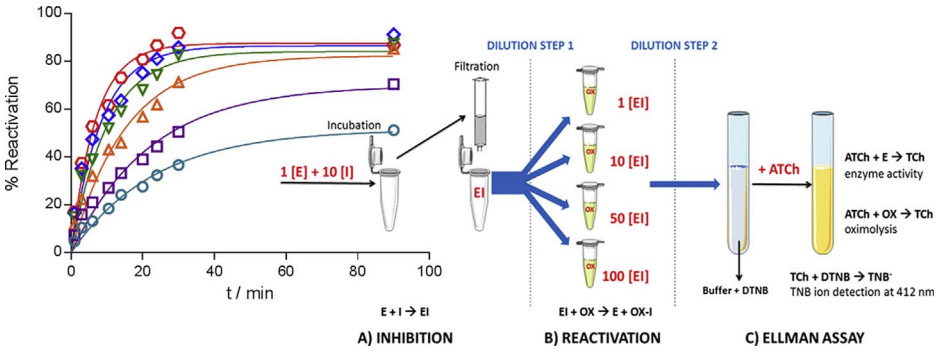
## GRAPHICAL ABSTRACT

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## ABSTRACT

Reactivation of acetylcholinesterase (AChE), an essential enzyme in neurotransmission, is a key point in the treatment of acute poisoning by nerve agents and pesticides, which structurally belong to organophosphorus compounds (OP). Due to the high diversity of substituents on the phosphorous atom, there is a variety of OP-AChE conjugates deriving from AChE inhibition, and therefore not only is there no universal reactivator efficient enough for the most toxic OPs, but for some nerve agents there is still a lack of any reactivator at all. The endeavor of many chemists to find more efficient reactivators resulted in thousands of newly-designed and synthesized oximes – potential reactivators of AChE. For an evaluation of the oximés reactivation efficiency, many research groups employ a simple spectrophotometric Ellman method. Since parameters that describe reactivator efficiency are often incomparable among laboratories, we tried to emphasize the critical steps in the determination of reactivation parameters as well as in the experimental design of a reactivation assay. We highlighted the important points in evaluation of reactivation kinetic parameters with an aim to achieve better agreement and comparability between the results obtained by different laboratories and overall, a more efficient evaluation of *in vitro* reactivation potency.

## 1. Introduction

Recent events in Syria regarding the use of the nerve agent sarin against civilians once again pushed the focus of attention on therapy against organophosphorus compounds (OP). In the event of OP poisoning, immediate medical intervention is vital due to the deadly effects caused by irreversible inhibition of acetylcholinesterase (AChE, EC 3.1.1.7), an enzyme essential in cholinergic neurotransmission. The currently applied therapy includes the anticholinergic drug atropine and an oxime reactivator of inhibited AChE, along with anti-seizure

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agents like diazepam (Dawson, 1994; Gray, 1984). Since there is neither a universal reactivator nor one with abundant efficiency, the search for more effective treatments of OP exposure than the currently used standard oximes (2-PAM, obidoxime or HI-6) is still ongoing.

The basic tool for the evaluation of oxime efficiency as a reactivator of OP-inhibited AChE is an assay based on the spectrometric Ellman method (Ellman et al., 1961). Nevertheless, even though the assay itself is simple, the kinetic parameters of reactivation are hardly comparable among laboratories due to various experimental approaches and data analysis. Over the past decades, several hundred compounds were tested in search for an effective reactivator of AChE inhibited by different OPs. The kinetic parameters obtained by these studies differed not only in the chemical differences of the tested compounds (*i.e.* counter ions), AChE species differences (Luo et al., 2007; Worek et al., 2002), the use of recombinant enzymes as opposed to native AChE (Kovarik et al., 2004; Lucić Vrdoljak et al., 2006; Maček Hrvat et al., 2016; Worek et al., 2012a), or experimentation at different temperatures, but also due to the differences in the experimental design and data analysis (Ashani et al., 1995; Kovarik et al., 2004; Luo et al., 2007; Maxwell et al., 2008; Musilek et al., 2011; Renou et al., 2016; Worek and Thiermann, 2011). Taking this into account, the search for an effective oxime reactivator is often difficult when one compares literature data for the structure activity relationship. Therefore, this study aimed to address the issue, mainly focusing on the experimental design and data analysis and on the example of sarin-inhibited human recombinant AChE reactivated by the standard oxime 2-PAM. Moreover, we singled out the key differences in the reactivation assay protocol found in the literature and emphasized experimental parameters that would help systematize the reactivation assay together with data analysis. This should enable interlaboratory comparisons of kinetic parameters determined by the evaluation of oxime-assisted reactivation of OP-inhibited AChE.

## 2. Materials and methods

### 2.1. Chemicals and enzyme

Sarin (NC Laboratory, Spiez, Switzerland) was diluted in isopropyl alcohol and further dilutions were made in water before use. 2-PAM (2-[(hydroxyimino)methyl]-1-methylpyridin-1-ium chloride), acetylthiocholine iodide (ATCh), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Recombinant human AChE, wild type (Cochran et al., 2011) was a kind gift from Professor Palmer Taylor, Skaggs School of Pharmacy and Pharmaceutical Sciences, UCSD, La Jolla, SAD.

### 2.2. Reactivation assay and evaluation of kinetics

AChE was incubated with a ten-fold excess of sarin until inhibition was greater than 95%. The inhibited enzyme was passed through a Sephadex G-50 spin column (Roche Diagnostic GmbH, Mannheim, Germany) to remove the excess of unconjugated sarin. After filtration and dilution in 0.1 M sodium phosphate buffer pH 7.4 containing 0.01% BSA, the inhibited enzyme was incubated with 2-PAM oxime in phosphate buffer (reactivation mixture, $R_{mix}$). At specified time intervals, an aliquot of $R_{mix}$ was diluted in phosphate buffer containing DTNB and upon ATCh addition, enzyme activity was measured according to Ellman method (Ellman et al., 1961). Final concentrations of ATCh and DTNB were 1.0 and 0.3 mM, respectively. An equivalent sample of uninhibited enzyme was passed through a parallel column, diluted to the same extent as the inhibition mixture, and control activity was measured in the presence of an oxime at concentrations used for reactivation. The concentration of the inhibited AChE in the $R_{mix}$ ranged from 0.2 to 22.2 nM, while 2-PAM concentrations were in the 4.4 to 1750 μM range.

Oxime-induced reactivation of the OP-AChE conjugate can be described through a two-step reaction (Kovarik et al., 2004) and proceeds according to Scheme 1:

$$EI + OX \underset{}{\overset{K_{ox}}{\rightleftharpoons}} [EI]\,[OX] \xrightarrow{k_2} E + I\text{-}OX \qquad k_r$$

where EI stands for phosphylated enzyme and OX for oxime, [EI][OX] is the reversible complex, E is the active enzyme and I-OX the phosphylated oxime. The $K_{ox}$ constant approximates the dissociation constant which is the reciprocal of the affinity of the [EI] to oxime, $k_2$ is the maximal first-order reactivation rate constant and $k_r$ is the overall second-order reactivation rate constant. Scheme 1 is defined by the equation:

$$\ln\frac{[EI]_0}{[EI]_t} = \frac{k_2\cdot[OX]}{K_{OX} + [OX]}\cdot t = k_{obs}\cdot t \tag{1}$$

where $[EI]_0$ and $[EI]_t$ represent the concentration of the phosphylated enzyme at time zero and at time t. $k_{obs}$ is the observed first order rate constant of reactivation at any given oxime concentration. The overall reactivation rate $k_r$ is the ratio:

$$k_r = \frac{k_2}{K_{OX}} \tag{2}$$

Experimental data were presented as percentage of reactivation (React) in time:

$$React = \frac{v(EI+OX)_t}{v(E+OX)}\cdot 100 \tag{3}$$

where $v_{(EI+OX)t}$ denotes the activity of the reactivated enzyme at time t and $v_{(E+OX)}$ stands for activity of uninhibited enzyme incubated with oxime (control). Since (100 − React) is equal to $100\cdot[EI]_t/[EI]_0$, one can relate the experimental data to Eq. (1), and $k_2$ and $K_{ox}$ were obtained from the plot $k_{obs}$ *vs.* [OX]. Control and reactivated AChE activities were corrected for oxime-induced degradation of ATCh (oximolysis).

At each oxime concentration, $k_{obs}$ was calculated by two methods: (a) non-linear regression (method A) utilized by one phase exponential increase in enzyme activity given in Eq. (4):

$$React = React_{max}\cdot(1 - e^{-k_{obs}\cdot t}) \tag{4}$$

where React stands for percentage of reactivation at given time, $React_{max}$ is the maximal percentage of reactivation and t is time of reactivation; and (b) linear regression of an initial part of the reactivation (method B).

Enzyme activity measurements were performed at 25 °C (or 37 °C) and at 412 nm, on the CARY 300 spectrophotometer (Varian Inc., Mulgrave, Australia) with a temperature controller. Prism software (Version 6, GraphPad Software, San Diego, CA, USA) was used for statistical analyses of results.

### 2.3. Acetylcholinesterase thermal stability

The enzyme incubated with OP to achieve at least 95% of inhibition was passed through a Sephadex G-50 spin column (Roche Diagnostic GmbH, Mannheim, Germany) and diluted in phosphate buffer pH 7.4 to a 0.7 μg/mL concentration. The uninhibited enzyme used as control was treated in the same way as the inhibited sample. Samples were divided in three sets and stored overnight at room temperature, 4 °C, and −16 °C. The sample's thermal stability was measured using a Prometheus NT.48 nanoDSF (NanoTemper technologies GmbH, Munich, Germany). The results are presented as fluorescence change against temperature.

## 3. Results and discussion

### 3.1. The design of the reactivation assays and selected concentration of reactants lead to an accurate determination of the reactivation efficiency

Generally speaking, a library of newly-synthesized compounds is to be tested for its reactivation potency and the screening should be simple and reliable with an aim to sort the compounds by their efficiency and identify promising reactivators for further testing. Basically, there are two general procedures for the screening. One procedure relies on the measuring of the recovery of inhibited cholinesterase activity once in 10, 30 or 60 min with one or two oxime concentrations that enable sorting compounds in terms of the reactivation percentage (Jun et al., 2008; Kuca et al., 2009; Musilek et al., 2011). Since reactivation depends on the time and oxime concentration, results evaluated in such way are almost incomparable with other similar screening studies where the same enzyme source is used, but the time of reactivation or studied oxime concentration are not the same. Although this procedure is helpful for indicating potential reactivators, to obtain more useful information on oxime efficiency, it has widely been accepted to use one oxime concentration for screening over longer times to approach the maximum of reactivation and determine the reactivation rate constant at the given oxime concentration $k_{obs}$ (Cochran et al., 2011017; Kovarik et al., 2013, 2015; Radić et al., 2013; Sit et al., 2011). However, to determine the reactivators' efficiency in terms of kinetic parameters, one should use a range of oxime concentration for the reactivation (Kovarik et al., 2004; Sharma et al., 2014). An additional source of confusion could be terminology because kinetic constants $k_2$ (maximum first order rate constant), $K_{OX}$ (dissociation constant) and $k_r$ (overall second-order reactivation rate constant) are often written as $k_r$, $K_D$ and $k_{r2}$, respectively.

For the reactivation assay, we used recombinant human AChE inhibited by sarin and a range of standard reactivator 2-PAM concentrations (4.4–1750 μM). In Table 1 we listed the determined reactivation parameters and parameters from several studies on sarin-inhibited AChE reactivated by 2-PAM (Luo et al., 2007; Sit et al., 2011; Worek et al., 2002) to indicate the differences resulting due to the source of AChE (human or animal, native or recombinant), evaluation of reactivation kinetics or experimental temperature. Further on, although reactivation experiments are usually performed at 25 °C or 37 °C, when comparing results from different studies one should keep in mind that reactivation kinetics is affected by temperature.

Nevertheless, in the last decade the source of the cholinesterase for the reactivation assays shifted from native (human, guinea-pig, etc.) such as erythrocyte preparations for AChE or plasma preparations for BChE to recombinant enzymes. This implies possible concentration variations of the OP-cholinesterase conjugate in the reactivation assay, which could possibly lead to a discrepancy in reactivation kinetic parameters and a difficulty in data comparison between studies. To test the potential effect of OP-cholinesterase conjugate concentration on reactivation rates, we designed a protocol in which the inhibited enzyme was diluted to obtained reactivation mixtures with various concentrations of inhibited-AChE (EI) (Fig. 1). The rationale for further dilution (Fig. 1, step 2) was (a) to reduce the inhibitory effect of the oxime on enzyme activity; (b) to reduce the oximolysis effect on enzyme activity, and (c) to quench the reactivation reaction. This protocol also ensures a sufficient number of $R_{mix}$ aliquots for the enzyme activity measurement, and the final EI concentration in the Ellman assay that corresponds to the control enzyme activity of about 0.1 $min^{-1}$ with 1 mM ATCh, in a volume of the Ellman assay mixture of 1 mL. Therefore, the final dilution of AChE for all four reactivation experiments was 4000 times. In other words, to acquire 0.2 nM EI, the volume of the reactivation mixture was 5 000 μL, while for 11.1 nM EI the volume was 100 μL to obtain 10 aliquots for activity measurements and the same final enzyme concentration in the Ellman assay. For the reactivation assay, we used recombinant human AChE, sarin and a range of the standard reactivator 2-PAM concentrations in order to determine kinetic parameters that would enable us to describe the effect of inhibited enzyme concentration, oximolysis, quenching and inhibition by the oxime on the reactivation kinetics.

Moreover, we came across modified reactivation assays where the inhibited enzyme was aliquoted and then frozen until reactivation experiments (Luo et al., 2007; Worek et al., 2012b). To test the enzyme structural stability with regard to storage temperature, the melting scan of uninhibited and inhibited AChE sample stored for 24 h at room temperature, 4 and −16 °C was monitored. The results showed that inhibited AChE was more stable than the control enzyme no matter what the temperature was (Fig. S1). Further, the variations in the initial fluorescence ratio, which may indicate different levels of sample degradation, were visible for both uninhibited and inhibited AChE samples kept overnight at −16 °C. Thus, the freezing of samples had a negative effect on enzyme structural stability and it could have led to an artifact in enzyme activity, even if the control sample was treated identically. In the need of sample freezing, it would be advisable to apply flash-freeze using liquid nitrogen to limit the denaturation induced by ice crystals formation and store the sample at −80 °C to limit the phosphylated enzyme ageing. Although there was no indication of degradation for samples kept overnight at room temperature or 4 °C, the drawback of a sample prepared 24 h prior to the reactivation assay is that it could result in a lower fraction of inhibited enzyme prone to reactivation due to the ageing reaction of the phosphylated AChE conjugate.

**Table 1**
Kinetic parameters: $k_2$ (maximum first order rate constant), $K_{OX}$ (dissociation constant) and $k_r$ (overall second-order reactivation rate constant) determined for sarin-inhibited AChE reactivation by 2-PAM.

| AChE | T/°C | Regression | $k_2$/$min^{-1}$ | $K_{OX}$/μM | $k_r$/$M^{-1}$ $min^{-1}$ |
|---|---|---|---|---|---|
| human recombinant | 25 | linear | 0.12 ± 0.001 | 262 ± 32 | 465 ± 40 |
| human recombinant | 25 | non-linear | 0.16 ± 0.009 | 190 ± 33 | 851 ± 114 |
| human recombinant | 37 | linear | 0.30 ± 0.021 | 255 ± 56 | 1168 ± 193 |
| human recombinant | 37 | non-linear | 0.86 ± 0.094 | 519 ± 141 | 1664 ± 296 |
| human recombinant[a] | 37 | non-linear | 1.1 ± 0.1 | 340 ± 160 | 3300 ± 1300 |
| human recombinant[b] | 22 | non-linear | 0.123 ± 0.032 | 266 ± 150 | 462 |
| human erythrocyte[c] | 37 | non-linear | 0.250 | 27.6 | 9060 |
| rabbit erythrocyte[c] | 37 | linear | 0.401 | 238.9 | 1680 |
| guinea-pig erythrocyte[c] | 37 | non-linear | 0.04 | 14.8 | 2700 |
| guinea-pig erythrocyte[b] | 22 | non-linear | 0.07 ± 0.022 | 342 ± 126 | 206 |

[a] Sit et al. (2011).
[b] Luo et al. (2007).
[c] Worek et al. (2002).

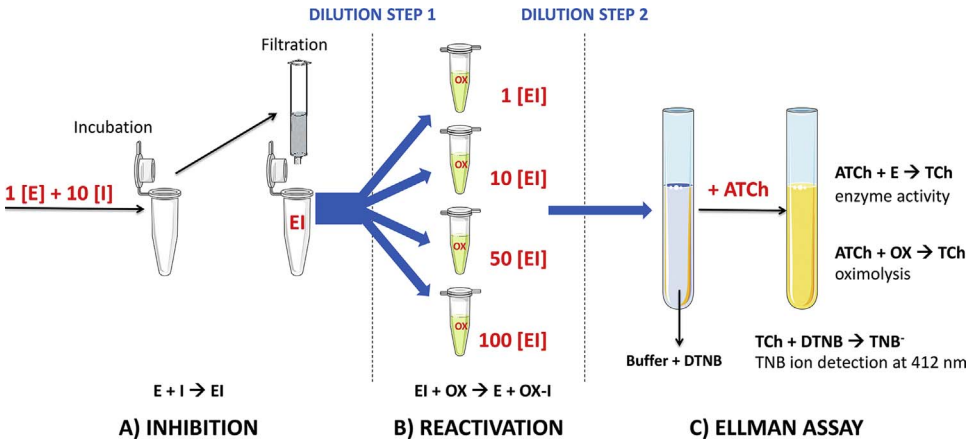


**Fig. 1.** General experimental design of the reactivation assay: A) AChE (E) incubation with a 10-fold inhibitor (I) excess until 95–100% of enzyme inhibition (~ 60 min), removal of inhibitor excess by filtration of inhibited enzyme (EI) and dilution in 0.1 M Na-phosphate buffer. B) EI aliquots were further diluted 2, 4, 20 and 200 times in a buffer containing the oxime (OX). C) at designated time points, aliquots of the reactivation mixture were further diluted 200, 20, 4 and 2 times in buffer with the Ellman reagent (DTNB). Enzyme activity was measured upon addition of the substrate acetylthiocholine (ATCh). The control sample was an uninhibited enzyme prepared in parallel as EI. The enzyme activity of E and EI was corrected for oximolysis.

### *3.2. The estimation of reactivator efficiency depends on the evaluation of kinetics parameters*

Reactivation kinetics is usually monitored over several reactivator concentrations to determine the relevant reactivation constants. There are two common evaluation methods for determining kinetic parameters – non-linear (Kliachyna et al., 2014; Radić et al., 2013; Sit et al., 2011; Worek et al., 2010) and linear regression (Kovarik et al., 2004, 2008, 2013, 2015; Maček Hrvat et al., 2016; Maxwell et al., 2008; Worek and Thiermann 2011). To test if the method is related to the determined oxime efficiency, we evaluated experimental data obtained on sarin-inhibited AChE reactivated by the range of the 2-PAM concentrations by both analysis methods in the way frequently found in literature, as shown in Fig. 2. The reactivation rate constant at given oxime concentration ($k_{obs}$) was determined with non-linear regression (Eq. (4); method A) and linear regression of an initial part of reactivation (method B), while the maximal first-order reactivation rate constant $k_2$, the dissociation constant $K_{ox}$, and the overall second-order reactivation rate constant $k_r$ were determined by Eqs. (1) and (2) from the secondary plot shown in Fig. 2C. Since the determined $k_{obs}$ differed between two evaluation methods, the discrepancy was reflected in the detailed reactivation parameters given in Table 2. Moreover, at the given EI concentration method A, provided approximately a 1.5 times

**Table 2**
Kinetic parameters of sarin-inhibited AChE reactivation by 2-PAM at 25 °C. The observed first order rate constant ($k_{obs}$) was determined by non-linear regression (method A) and linear regression (method B). Reactivation parameters: $k_2$ (maximum first order rate constant), $K_{OX}$ (dissociation constant) and $k_r$ (overall second-order reactivation rate constant) were evaluated from at least three experiments (means ± SEM).

| React$_{mix}$ [EI]/ nM | Parameter | Method A | Method B | Ratio A *vs.* B |
|---|---|---|---|---|
| **0.2** | $k_2$/min$^{-1}$ | 0.89 ± 0.092 | 0.67 ± 0.064 | 1.33 |
| | $K_{OX}$/μM | 150 ± 47 | 300 ± 60 | 0.50 |
| | $k_r$/M$^{-1}$ min$^{-1}$ | 5900 ± 140 | 2200 ± 260 | 2.70 |
| **2.2** | $k_2$/min$^{-1}$ | 0.39 ± 0.026 | 0.23 ± 0.014 | 1.70 |
| | $K_{OX}$/μM | 300 ± 52 | 250 ± 38 | 1.20 |
| | $k_r$/M$^{-1}$ min$^{-1}$ | 1300 ± 150 | 910 ± 94 | 1.42 |
| **11.1** | $k_2$/min$^{-1}$ | 0.16 ± 0.009 | 0.12 ± 0.001 | 1.33 |
| | $K_{OX}$/μM | 190 ± 33 | 260 ± 32 | 0.73 |
| | $k_r$/M$^{-1}$ min$^{-1}$ | 850 ± 114 | 470 ± 40 | 1.83 |
| **22.2** | $k_2$/min$^{-1}$ | 0.13 ± 0.008 | 0.08 ± 0.004 | 1.63 |
| | $K_{OX}$/μM | 270 ± 53 | 300 ± 54 | 0.90 |
| | $k_r$/M$^{-1}$ min$^{-1}$ | 490 ± 73 | 250 ± 34 | 1.93 |

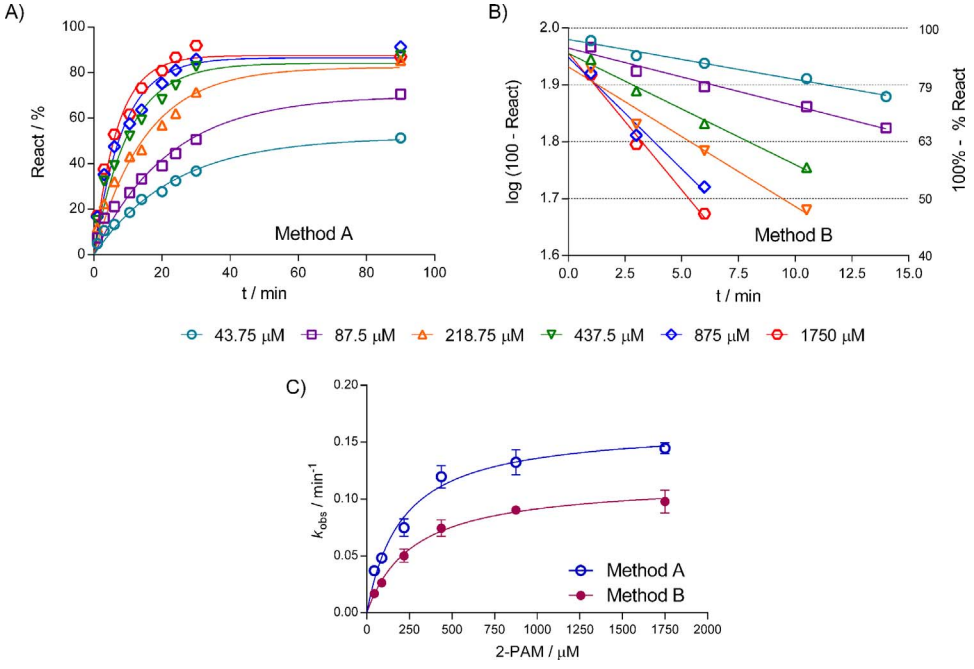


**Fig. 2.** Representative reactivation experiment of the sarin-inhibited AChE (11 nM) by 2-PAM at 25 °C analyzed by: A) non-linear regression, and B) linear regression to determine the observed first-order reactivation rate ($k_{obs}$). C) Comparison of two evaluation methods and their effect on the reactivation constants.

higher $k_2$ values and an up to 3 times higher $k_r$ values than method B. Along with different datasets used (for method B dataset is truncated to the first time points), this difference may have occurred because method A precedes the dependence of reactivation maximum with oxime concentrations despite a very common appearance of the different reactivation plateaus that are still unexplained − could be due to the reinhibition of AChE by phosphylated oxime, ageing, etc. (Ashani et al., 2003; Leader et al., 1999). Thus, a non-linear regression evaluation of $k_{obs}$ depends greatly on the maximal reactivation ($React_{max}$), which implies an overestimation of the $k_{obs}$ rate when reactivation ends on different plateaus. To circumvent the effect of different reactivation maximums at a given oxime concentration, as well as the resulting polyphasic reactivation curve (Fig. 2A), a reliable determination of the first-order reactivation rate $k_{obs}$ is the linearization of the initial part of the reactivation curves. Moreover, this method of analysis can be applied even if the reactivation is not monitored for longer time periods, which is very convenient in the case of high ageing rates of the phosphylated enzyme. Nevertheless, we should emphasize that the evaluation of constants with method A and method B would result in a similar constant $k_{obs}$ if the same experimental points were used for regressions as is the case when all reactivation curves are monophasic and there is no dependency of the reactivation maximum on oxime concentration.

It is important to note that in case of poor reactivation (*e.g.* below 20%), the determination of reactivation kinetic constants is pointless, and then the reactivation efficacy can be sorted by maximal reactivation ($React_{max}$) and the time in which the maximum is reached (Gašo-Sokač et al., 2010). On the other hand, in the case of fast reactivation rates one can use a continuous reactivation assay (Kovarik et al., 2007; Worek et al., 2002) which in fact mimics the *in vivo* conditions where we can expect a high substrate concentration in the case of AChE inhibition and that could potentially affect the reactivation rate.

Regardless of the evaluation method we used, the reactivation constants given in Table 2 indicated their high dependency on the enzyme concentration in reactivation mixtures. To address this effect, we related the enzyme concentration with the $k_2$, $k_{obs}$ and 2-PAM concentration (Fig. 3). Both $k_{obs}$ and $k_2$ exponentially decreased with EI concentration, *i.e.* $k_2$ was 7–8 times higher at 0.2 nM enzyme concentration than that at 22.2 nM (depending on the method of analysis). However, it is important to note that there was no effect on the achieved maximal reactivation, which remained the same at the given EI concentration in reactivation. It is also evident from Fig. 3A that the effect of enzyme concentrations above 5 nM on the reactivation rates is almost negligible, and that therefore the enzyme concentration in the reactivation assays should be adjusted to this range to provide a comparable estimation of reactivation rates. It is interesting to note that in our previous studies with erythrocytes, the AChE concentration in reactivation also remained within that range (7 nM) (Katalinić et al., 2016; Kovarik et al., 2008).

### 3.3. Importance of the final dilution of reactivation mixture in the ellman assay

Oximes, besides their reactivation potency, act as reversible inhibitors of cholinesterases (Katalinić et al., 2017; Kovarik et al., 2008; Odžak et al., 2007), and are capable on the other hand to spontaneously degrade the substrate in Ellman assay; a reaction known as oximolysis (Šinko et al., 2007). Therefore, it is advisable to measure control enzyme activity in the presence of the oxime to separate the AChE inhibition from the reactivation reaction as well as from the non-enzymatic reaction of the oxime, DTNB and ATCh. The enzyme activity needs to be corrected for oximolysis, especially when the reactivation is monitored with higher oxime concentrations, because the oximolysis could account for more than 20% of the absorbance signal. Without a control that includes an oxime at a given concentration and a correction of the absorbance for oximolysis, both effects on enzyme activity become part of observed reactivation rates $k_{obs}$ that lead to miscalculated reactivation rate constants. In addition, it should be mentioned that oximolysis rate increases with temperature and 2-PAM oximolysis at 37 °C is approximately 30% higher than at 25 °C.

Furthermore, since this study employed a wide range of 2-PAM concentration together with four concentrations of the inhibited enzyme in a reactivation mixture, we could address the importance of the quenching of the reactivation reaction in the Ellman assay. In other words, the quenching phenomenon occurs as a result of sufficient reactivation mixture dilution. In our reactivation protocol, two-fold dilution of the 0.2 nM inhibited enzyme was not sufficient for quenching the reactivation reaction, particularly at higher oxime concentrations when the reactivation with 2-fold diluted oxime was still taking place in the Ellman assay. This resulted in the artifact of a higher reactivation percentage.

Observing the final dilution step in Ellman assay with regard to the concentration of the inhibited enzyme, we came to the finding that the reactivation mixture dilution in the Ellman assay has to be more than 20-fold in order to quench the reactivation reaction. Moreover, in most cases, both oxime effects on enzyme activity − oximolysis and reversible inhibition − could be attenuated by a 100-times dilution factor in the Ellman assay.

### 3.4. The effect of the measured time-scale − a theoretical approach

Some studies apply an end-point approach in the AChE reactivation meaning that AChE activity is measured just once to determine not only the reactivation efficiency in terms of the percentage of reactivation but also to evaluate the kinetic constants of reactivation (Kuca et al., 2003, 2005). To describe the advantages or disadvantages of such an approach, we performed a theoretical study using our results on the reactivation course of sarin-inhibited AChE (11.1 nM) with 2-PAM at 25 °C (cf. Table 2, method B,) and obtained $k_{obs}$ at the given 2-PAM concentrations, while the maximal reactivation was set to 100%. The simulation is given in Fig. 4.

Exponential reactivation curves were generated using Eq. (4) up to 90 min at given 2-PAM concentrations. At arbitrarily selected times (3 to 90 min), the reactivation was related with a given 2-PAM concentration that corresponds to an end-point approach, and the apparent kinetic parameters, $React_{max}$ and $K_{OX}$, were re-calculated from the hyperbolic correlation given at panel 4B. As we expected, the values of the apparent parameters exponentially depended on the time of taking out the aliquot of reactivation mixture for measuring enzyme

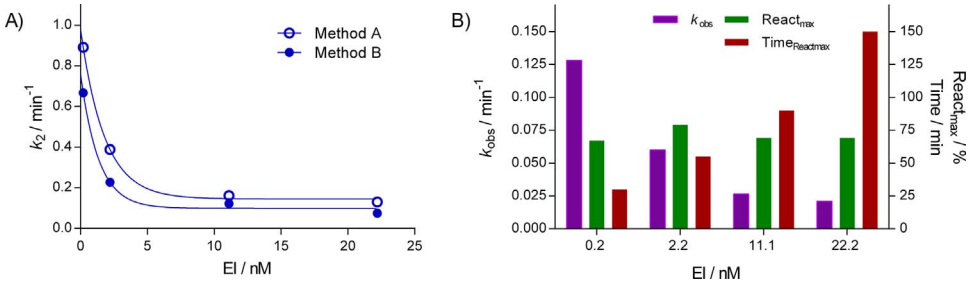


**Fig. 3. A)** Dependence of maximal reactivation rate $k_2$ on the EI concentration in the reactivation mix, analyzed by method A and B. B) Reactivation of 0.2–22.2 nM sarin-AChE conjugates with 87.5 μM 2-PAM. Observed reactivation rate ($k_{obs}$, analyzed by method B), maximal percentage of reactivation ($React_{max}$) and time in which it was achieved are presented as mean of up to four experiments.

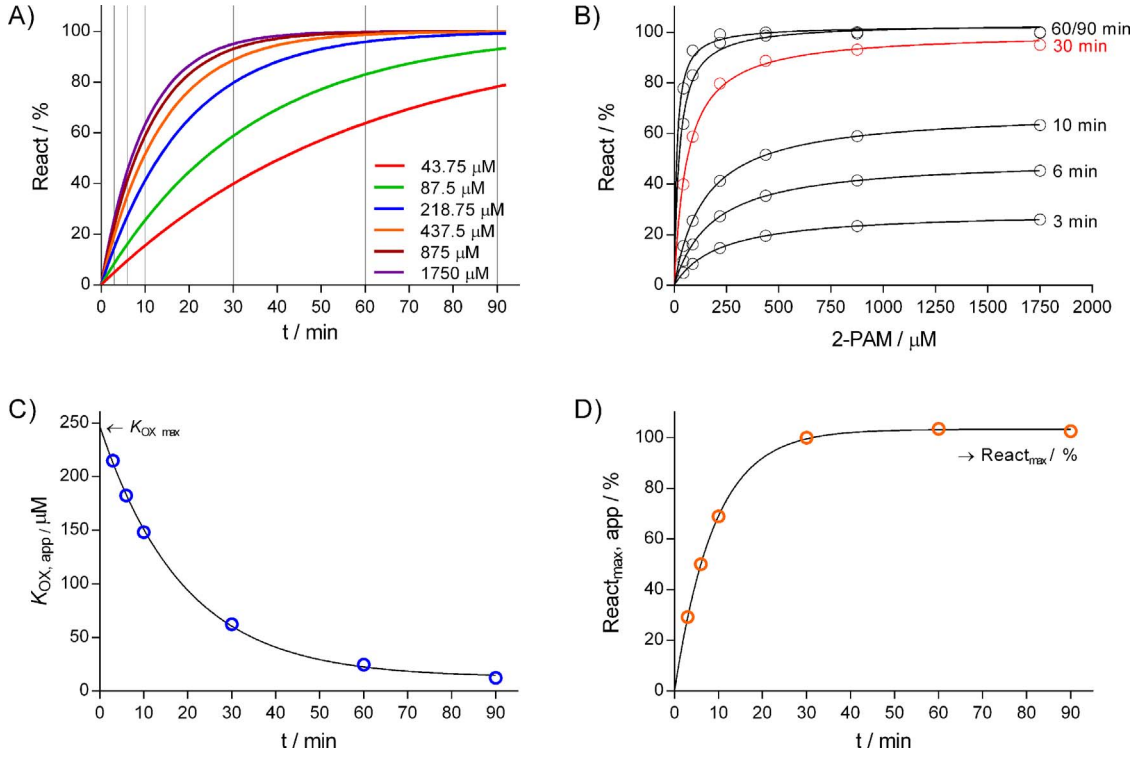


**Fig. 4.** Simulated course of reactivation for 11.1 nM AChE-sarin conjugate with 2-PAM at 25 °C using Eq. (4). The kinetic parameters used for the analysis are listed in Table 2 (row 3, method B).

activity − $React_{max}$ increased and apparent $K_{OX}$ decayed. Therefore, since reactivation kinetics parameters depend on evaluation time, an end-point approach in the evaluation of reactivator efficiency may result in an artifact, *i.e.* in case of efficient reactivators ($t_{½}$ below 1 min) with $React_{max}$ approaching 100% but with significantly lower $K_{OX}$ value compared to the true $K_{OX}$. Other scenarios in case of a poor reactivator ($t_{½}$ above 30 min) would result in a $React_{max}$ significantly lower than 100% due to a slow reactivation of the OP-AChE conjugate, and the $K_{OX}$ value would be closer to true $K_{OX}$.

Nevertheless, the end-point approach of reactivator efficiency evaluation is acceptable for screening a large number of reactivators to sort them in terms of the percentage of reactivation. For determination of kinetic parameters $k_2$, $k_r$ and $K_{OX}$ which are supposed to be "independent" from the experimental design, detailed reactivation kinetics should be set with a concentration range of the reactivator.

## 4. Conclusions

The estimation of reactivation efficiency depends on experimental design and data analysis, but to get reliable and comparable kinetic constants among laboratories and different experimental approaches, the reactivation assay needs to include monitoring side reactions − oximolysis, reversible inhibition, and adequate dilution in Ellman reaction in order to effectively quench the reactivation reaction. The presentation of the results should be accompanied with the details on the source of the enzyme and its concentration (dilution factor in case of native enzyme), evaluation method and experimental temperature.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.toxlet.2017.11.022.

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