

ORIGINAL ARTICLE

ANALYSIS OF NERVE AGENTS THROUGH A MODIFIED METHOD OF CHOLINESTERASE ASSESSMENT USING ARTIFICIAL NEURONAL NETWORKS

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Received 15th May 2017.

Revised 9th June 2017.

Published 8th September 2017.

Summary

A simple colorimetric biosensor, which uses the modified Ellman's reaction, enables selective analysis of nerve agents based on a different ability of bispyridinium oximes to reactivate enzyme-inhibitor complexes in the phase before dealkylation. The analysis was made based on spectral data of reflectance of the surface of a cotton cloth reaction zone of biosensor with immobilized and stabilized enzyme after inhibition and subsequent reactivation. The evaluation of measured data was made based on a method of artificial neuronal networks. The individual inhibitors from the groups of three nerve agents were identified: sarin, soman, tabun, cyclosarin, agent VX and its Russian analogue, R-33.

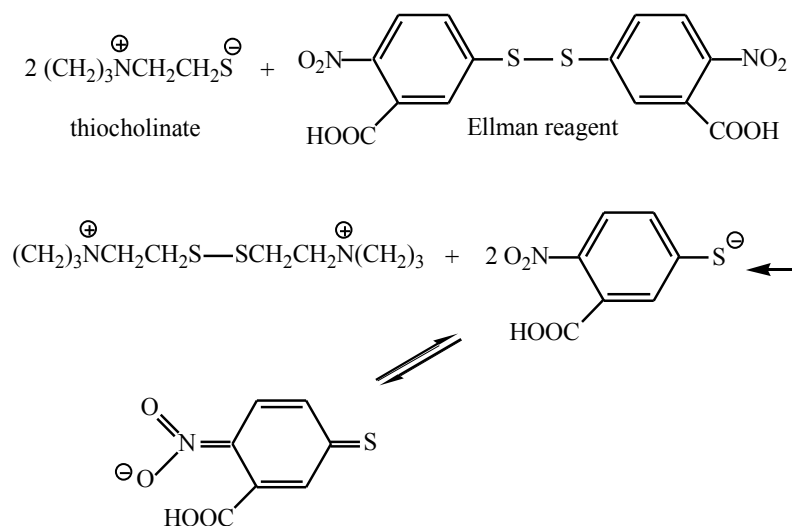
Key words: Ellman's reagent; chemical warfare agent; colorimetric biosensor; acetylcholinesterase reactivator; neural analysis

INTRODUCTION

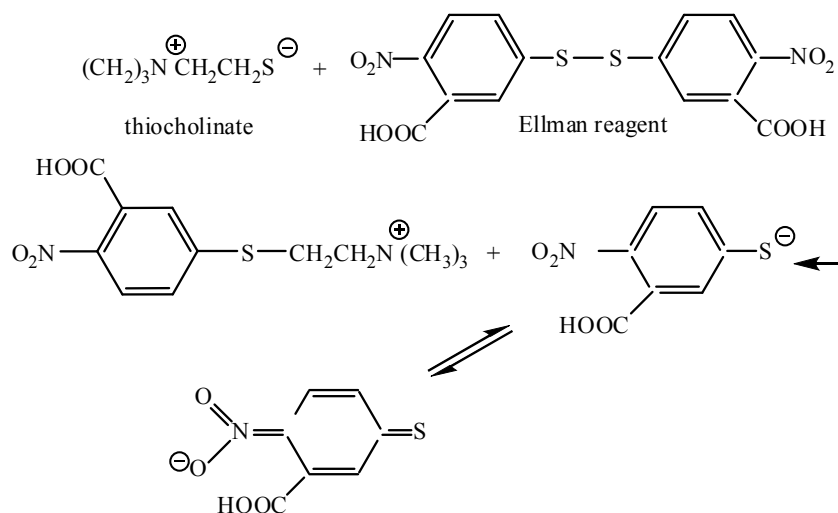
The nerve agents can be detected by many ways, for example by means of commonly available group reagents which are commercially available. These include detection papers, tubes, foils, cloths and others. Some of those reagents make use of a biochemical reaction simplified, which is advantageous because it displays high sensitivity, which is even several times higher compared to methods based on chemical or physical principle. The reaction is based on the principle of hydrolase inhibition which specifically hydrolyses the appropriate substrate. The hydrolases, which are used, include enzyme acetylcholinesterase, also called specific cholinesterase, or butyrylcholinesterase (the nonspecific cholinesterase), as acetyl- or butyrylcholine substrate. [1]

The biochemical principle is based on identification of the presence of inhibitor of an appropriate enzyme. None of the above mentioned detection means enables its selective identification. This could be possible through DeteHit, a detector of nerve agents. [2] There the biochemical reaction is modified to detection of the presence of sulphhydryl groups by Ellman. [3] The detector is made up of a cotton cloth with immobilized and stabilized acetylcholinesterase and a detection paper on which the acetylthiocholine iodide and Ellman's reagent – 5,5'-dithio-bis(2-nitrobenzoic) acid – are laid. The substrate hydrolysis, catalyzed by acetylcholinesterase into thiocholine, is indicated by development of an intensive yellow coloring caused by generation of a reduced form of the Ellman's

chromogenic reagent. There are two possible mechanisms. Reactions are shown in Scheme 1 and 2. [1,4] The presence of the inhibitor is detected by white or lightly yellow coloring of the cloth, depending on the degree of enzyme inhibition.



Scheme 1. Ellman's reaction I



Scheme 2. Ellman's reaction II

Biochemical reaction, e.g. Ellman's modification, has not been a selective method up to now. It was not possible to differentiate among individual nerve agents. For selective identification we have suggested using the detector of nerve agents in connection with nucleophilic reagents – acetylcholinesterase reactivators. Selective identification of individual nerve agents is enabled, even before the development of the non-reactivable form of the phosphorylated enzyme. Nucleophilic reagents restore the function of the enzyme by unbinding the inhibitor from its active center. In clinical practice compounds based on pyridinium aldoximes are used. [5] Their efficacy varies and depends on many factors such as structure, number and placement of functional groups and cationic sites, number

of pyridinium nuclei etc., but also the type of the inhibitor. [6-8] Thanks to those influences there is no broad-spectrum reactivator which would be able to react effectively with an enzyme inhibited by any nerve agent in human organism. This poses difficulties in antidotal therapy, but it can be used for nerve agents identification. Exactly the different efficacy of these oxime compounds compared to characteristic enzyme-inhibitor complexes results in the already mentioned change to the intensity of color of the biosensor. From the original white back to yellow owing to arising products of the substrate hydrolysis. The previous measurements made after comparing the yellow coloring of the surface of the cloth after reactivation had shown that visual differentiation was virtually impossible. Differences in the intensity of coloring are not perceivable with a human eye. [9] This can be done with high sensitivity by detecting the reflectance of the color surface of an impregnated cloth.

It was apparent that there was a need for finding a suitable method to identify a nerve agent correctly. [10-13] Neuronal networks are used in cases when it is not possible to describe mathematically all the relations and connections which influence the process observed in solution of the given problem. It also applies to cases when we succeed in setting up the mathematical model but it is so complicated that the pertinent algorithmization of the task is almost impossible. This especially applies to complicated and highly non-linear systems.

ANN are applied in ever greater number of diverse fields such as reconstructions of damaged patterns, classification tasks, predictions, approximations, extrapolations, recognition of patterns and other areas. They are utilized in various areas of science and industry. [14-17]

An inspiration for ANN was the structure of functional cells of the nervous system of living organisms – neurons. Neuron is thus the basic construction unit of neuronal networks. It fulfills the function of a calculating unit processing inputs, outputs and internal states. [18]

The basic ANN types include Multilayer Perceptron Neural Network (MLP), Radial Basic Function network (RBF), Cohen's network, linear network and Bayesian networks. The network type selection depends on the type of task solved and on the character of data. For different tasks different ANN types are suitable. [10] Many times the selection of an appropriate type is difficult, it requires experience and often it is needed to experiment with several types of networks.

We have chosen MLP Network for our task. It is the best-known and mostly used neuronal network including multilayer perceptron network where perceptron is the basic element. The activation function of perceptron is most often the logistic function – sigmoid. MLP is a network with a teacher, i.e. besides input values, training patterns must also include values of appropriate corresponding outputs. The network consists of several perceptron layers – input layer, output layer and several hidden layers. Perceptrons of individual adjacent layers are interconnected, i.e. the output of one neuron is distributed into outputs of all perceptrons of the next layer. For the network to function properly the weights of network must be set appropriately. The way how to set those weights is the subject of network learning. For MLP we have a number of sophisticated methods of learning. One of the basic methods of learning is the method of error backpropagation. After learning, the network is able to recall patterns, to estimate further development and, in our case, to classify the output variable correctly. [19]

MATERIAL AND METHODS

Chemicals

The cloth with immobilized porcine brain acetylcholinesterase [EC 3.1.1.7] and the indicator paper with acetylthiocholine iodide and 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB), CAS 69-78-3 were provided by ORITEST, Ltd., Prague, Czech Republic.

Nerve agents: isopropyl-methylphosphonofluoridate – sarin, GB, CAS 107-44-8; 1,2,2-trimethylpropyl-methylphosphonofluoridate – soman, GD, CAS 96-64-0; O-cyclohexyl-methylphosphonofluoridate – cyclosarin, GF, CAS 329-99-7; ethyl- dimethylphosphoramidocyanidate – tabun, GA, CAS 77-81-6; S-[2-(diisopropylamino)ethyl]-O-ethylmethylphosphonothioate – VX agent, VX, CAS 50782-69-9 were prepared in VOZ

(Military Repair Plant) 072, Zemianské Kostolany, Slovak Republic. S-[2-(diethylamino)ethyl]-O-isobutylmethylphosphonothioate agent – R-33 agent was prepared in Military Research Institute, Brno, Czech Republic. The purity of nerve agents ranged between 60 - 65 %. Purity was evaluated by GC-MS. For inhibition of the immobilized and stabilized enzyme organophosphates in concentrations of 10^{-5} – 10^{-4} mg.mL⁻¹ were used.

Reactivators: *N,N'*-trimethylenebis(4-pyridiniumaldoxime) dibromide – TMB-4, purity 95 %, and 1-(2-hydroxyaminomethylpyridinium)-3-(4-karbamoyl pyridinium)-2-oxapropane dichloride – HI-6, purity 97 %, were prepared by Kroupa-Balex, Ltd., Pardubice, Czech Republic.

Working procedure

Decreased activity was predetermined in set of measurements for given concentration of inhibitor just before reactivation. The cloth with immobilized and stabilized acetylcholinesterase was exposed in a water solution of a nerve agent for 2 minutes so as to inhibit the enzyme for 10 up to 20 % of the original activity. Measurements were provided for every new batch of cotton cloth with immobilized enzyme in certain conditions.

For assessment of reactivation efficiency, the cotton cloth was exposed in a water solution of a nerve agent for 2 minutes. After that the cloth was flushed with 5 mL of distilled water in order to remove the surplus of the inhibitor. Subsequently it was dipped into water solution of a nucleophilic reagent. The concentration of these reagents was 0.1 mg.mL⁻¹ for the reactivation time of 15 minutes. After the given time of reactivation, the cloth was taken out of the solution and again flushed with distilled water. For 1 minute an indicator paper with substrate and chromogenous reagent was applied. Depending on the degree of reactivation there was a reaction of the produced thiocholine with Ellman's reagent, which resulted in a different intensity of yellow coloring. The Ultra Scan XE spectrometer was used for measuring the reflectance of a beam from the cloth reaction zone within the wavelength range of 380 – 750 nm as from the 20th second for 2 minutes at 10-second intervals. Measurement conditions have been: reflectance spectral data type, 10 nm reflectance resolution, absolute display mode, D 65 illuminant and Fw MI illuminant.

Devices and software used

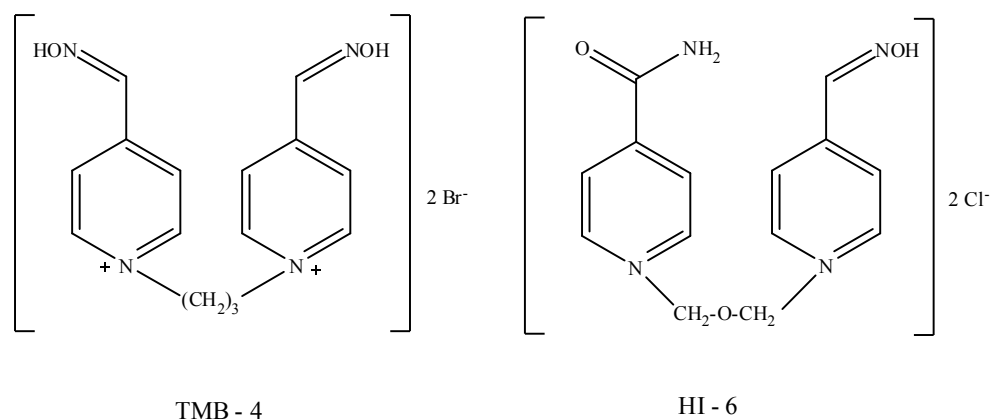
ULTRA SCAN XE* spectrophotometer, Hunter Lab, Reston, VA, USA. Universal Software (4.01 version). For processing the measured data, the analysis by means of Artificial Neuronal Networks (ANN) was selected. Statistical software Statistica, 9 version, StatSoft CR, Prague, Czech Republic.

RESULT AND DISCUSSION

Changes in intensity of coloring of a detection cloth with immobilized enzyme after inhibition through nerve agents and subsequent reactivation through a set of mono- and bispyridinium aldoximes were measured. For this purpose the best-known reactivators were selected, many of them used in antidotal therapy. Pralidoxime and methoxime and also HI-6 reactivators were the basis for these series. Out of the originally studied concentrations within the range of 0.5 - 0.01 mg.mL⁻¹ as the most representative the concentration of 0.1 mg.mL⁻¹ was chosen, with the reactivator reaction time of 15 minutes out of the originally measured 10, 15, 20. Differences in efficacy between 15 and 20 minutes are not significant. Sarin, soman, tabun, cyclosarin, VX and R-33 inhibitors were used.

To differentiate nerve agents based on the reactivation efficacy is problematic, because of the majority reactivators' low efficacy. This phenomenon was observed especially with acetylcholinesterase inhibited by soman, tabun, VX and R-33 agents where only less significant differences in efficacy of individual oximes were detected. [20,21] In case of soman, quick development of a dealkylated form of phosphorylated enzyme is probable. At this stage the influence of nucleophilic reagents is not efficient anymore. According to the study [22] the enzyme inhibited by tabun is difficult to reactivate thanks to the existence of a lone electron pair on amidic nitrogen which makes the nucleophilic reaction of reactivators difficult and according to the crystallographic study [23] this complex is influenced by other residues in the active enzyme center which cause structural changes leading to reduction of the size of this cavity. This fact may lead to inability of more voluminous molecules of reactivators to penetrate the point of inhibitor bond in the esteratic center.

For the above mentioned reasons only the quantification of efficacy of individual reactivators is not sufficient for identification of one nerve agent out of a group of three agents and so the statistical evaluation by means of artificial neuronal networks was approached. Values of reflectance within the whole spectrum of wavelengths in the visible region of electromagnetic radiation, i.e. from 380 to 750 nm, were measured. The measurements were taken throughout the course of biochemical reaction at least within 2 minutes always at regular intervals. This enabled to cover qualitatively all the changes in intensity of coloring of the cloth reaction zone. These changes, based on the neural analysis, seem to be characteristic for the given type of inhibitor and they are striking within the first minute of the course of Ellman's reaction.



Scheme 3. Acetylcholinesterase reactivator structure – TMB-4 (*n,n'*-trimethylene bis(4-pyridiniumaldoxime) dibromide) and HI-6 (1-(2-hydroxyaminomethylpyridinium)-3-(4-karbamoylpyridinium)-2-oxapropane dichloride)

For identification of the inhibitor as an output parameter the classification method of problem solution with default setting for the MLP network was used. The input data for this network include the time course of spectral values of reflectance of the surface of a cloth with immobilized enzyme which was inhibited and subsequently reactivated by HI-6 or TMB-4 bispyridinium aldoximes. Their structure is shown in Scheme 3. Selection of these reactivators for identification of nerve agents was made on the base of their good efficacy in reactivation of acetylcholinesterase inhibited with sarin. Results were published in (Hoskovicová, 2006). [9]

Through this method it is possible to differentiate the given inhibitor in the event that the sample contains only one agent out of the given group of three for which the analysis was made. The results in tables show the high accuracy of the correct inhibitor identification which ranges from 98 up to 100 % of congruently identified cases. The results of neural analysis for groups of three inhibitors and HI-6 and TMB-4 reactivators ($0.1 \text{ mg}\cdot\text{mL}^{-1}$) are presented in tables below.

Table 1. The results of neural analysis for groups of three inhibitors and HI-6 reactivator $0.1 \text{ mg}\cdot\text{mL}^{-1}$, reactivation time of the enzyme-inhibitor complex 15 minutes.

Agents	MLP network characteristics ^a	Total of cases ^b	Correctly identified cases ^c	Incorrectly identified cases ^d	Correctly identified, % ^e
GA:GF:R-33	40:58-10-3:1	149:172:99	149:170:98	0:2:1	99.3
GA:GF:VX	40:58-8-3:1	149:173:112	149:172:110	0:1:2	99.3
GA:R-33:VX	40:58-8-3:1	149:100:115	148:98:115	1:2:0	99.2
GA:GB:GF	40:58-8-3:1	152:202:164	151:202:163	1:0:1	99.6
GB:GA:R-33	40:58-18-3:1	149:200:99	147:200:97	2:0:2	99.1
GB:GA:VX	40:58-22-3:1	201:149:112	201:149:110	0:0:2	99.6

Agents	MLP network characteristics ^a	Total of cases ^b	Correctly identified cases ^c	Incorrectly identified cases ^d	Correctly identified, % ^e
GB:GD:GA	40:58-11-3:1	200:94:154	200:93:154	0:1:0	99.6
GA:GF:R-33	40:58-8-3:1	196:94:172	195:93:171	1:1:1	99.4
GB:GD:R-33	40:58-20-3:1	195:95:102	195:95:101	0:0:1	99.7
GB:GD:VX	40:58-10-3:1	197:94:115	197:94:113	0:0:2	99.5
GB:GF:R-33	40:58-24-3:1	196:172:94	195:172:93	1:0:1	99.6
GB:GF:VX	40:58-14-3:1	195:170:111	195:170:110	0:0:1	99.8
GB:R-33:VX	40:58-8-3:1	197:94:115	197:94:113	0:0:2	99.5
GD:GA:GF	40:58-8-3:1	94:154:172	94:152:172	0:2:0	99.5
GD:GA:R-33	40:58-22-3:1	94:155:101	94:153:97	0:2:4	98.3
GA:GD:VX	40:58-8-3:1	154:96:105	152:96:104	2:0:1	98.2
GD:GF:R-33	40:58-23-3:1	94:169:101	92:169:99	2:0:2	98.9
GD:GF:VX	40:58-9-3:1	95:167:116	95:166:114	0:1:2	99.2
GD:R-33:VX	40:58-23-3:1	95:101:112	94:100:112	1:1:0	99.4

Problem solution through the classification method with default setting for the multilayer perceptron neural (MLP) network.

^a MLP network characteristics = identification of the number of neurons in individual layers – input : hidden : output;

^b Total of cases = training set of experimental data, i.e. values of reflectance of the surface of a cloth depending on development of Ellman's reaction in time and wavelength;

^c Correctly identified cases = number of congruently determined cases of agent (inhibitor) identification with cases of trained network;

^d Incorrectly identified cases = number of incorrectly determined cases of agent (inhibitor) identification with cases of trained network;

^e Correctly identified, % = percentage expression of congruently set cases.

Table 2. The results of neural analysis for groups of three inhibitors and TMB-4 reactivator 0.1 mg.mL⁻¹, reactivation time of the enzyme-inhibitor complex 15 minutes.

Agents	MLP network characteristics ^a	Total of cases ^b	Correctly identified cases ^c	Incorrectly identified cases ^d	Correctly identified, % ^e
GA:GF:R-33	40:58-8-3:1	85:70:97	85:69:97	0:1:0	99.6
GA:GF:VX	40:58-24-3:1	84:71:55	84:70:55	0:1:0	99.5
GA:R-33:VX	40:58-18-3:1	86:97:55	86:97:55	0:0:0	100
GA:GB:GF	40:58-9-3:1	85:97:70	85:96:70	0:1:0	99.6
GB:GA:R-33	40:58-15-3:1	85:98:97	84:98:96	1:0:1	99.3
GB:GA:VX	40:58-25-3:1	86:96:56	85:96:70	0:1:0	99.6
GB:GD:GA	40:58-19-3:1	100:70:82	100:69:82	0:1:0	99.6
GB:GD:GF	40:58-24-3:1	99:71:68	97:71:68	2:0:0	99.2
GB:GD:R-33	40:58-8-3:1	86:71:99	85:69:99	1:2:0	98.9
GB:GD:VX	40:58-24-3:1	97:71:56	97:71:55	0:0:1	99.6
GB:GF:R-33	40:58-22-3:1	102:67:97	102:67:97	0:0:0	100
GB:GF:VX	40:58-15-3:1	95:71:58	95:71:57	0:0:1	99.6
GB:R-33:VX	40:58-24-3:1	100:96:56	100:94:56	0:2:0	99.2
GD:GA:GF	40:58-8-3:1	82:71:71	82:71:71	0:0:0	100
GD:GA:R-33	40:58-9-3:1	82:72:100	82:71:100	0:1:0	99.6
GA:GD:VX	40:58-20-3:1	81:69:60	81:68:59	0:1:1	99.0
GD:GF:R-33	40:58-10-3:1	71:68:99	71:68:99	0:0:0	100

Agents	MLP network characteristics ^a	Total of cases ^b	Correctly identified cases ^c	Incorrectly identified cases ^d	Correctly identified, % ^e
GD:GF:VX	40:58-22-3:1	69:71:56	68:71:56	1:0:0	99.5
GD:R-33:VX	40:58-8-3:1	71:99:54	69:97:54	2:2:0	98.2
GF:R-33:VX	40:58-8-3:1	71:97:56	71:96:56	0:1:0	99.6

Problem solution through the classification method with default setting for the multilayer perceptron neural (MLP) network.

^a MLP network characteristics = identification of the number of neurons in individual layers – input : hidden : output;

^b Total of cases = training set of experimental data, i.e. values of reflectance of the surface of a cloth depending on development of Ellman's reaction in time and wavelength;

^c Correctly identified cases = number of congruently determined cases of agent (inhibitor) identification with cases of trained network;

^d Incorrectly identified cases = number of incorrectly determined cases of agent (inhibitor) identification with cases of trained network;

^e Correctly identified, % = percentage expression of congruently set cases.

CONCLUSIONS

Differentiation of individual organophosphates from groups of three agents was done using the colorimetric biosensor with immobilized enzyme after reaction with oxime TMB-4 and HI-6 reactivators. These reactivators were chosen for their practical use in clinical practice. *In vitro* the efficacy of individual oximes, except for sarin, is close with the majority of agents. On the basis thereof it is not possible to differentiate the individual inhibitors. Reliable differentiation was achieved by comparing spectral data of reflectance of the surface of a cotton carrier. The evaluation was made through the classification method of artificial neuronal networks of the statistical software Statistica. It has been shown that the method of artificial neuronal networks is suitable for analysis of spectral data. Neuronal networks provide very good results especially in this case which is characterized by a high degree of non-linearity and complexity of mathematical description. Thus in analysis of organophosphates it is possible to select one inhibitor from the potential group of three with high probability, with little time demandingness.

This method, e.g. modified Ellman's method, in connection with artificial neuronal networks, enables selective differentiation of single nerve agents.

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