The Synthesis and Toxicological Characterization of Neurotoxic Chemical Agents Simulants

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Neurotoxic warfare chemical agents (CWA/NA) induce major toxicological effects to the affected personnel. Their increased toxicity justifies the necessity of developing analytical methods for diagnosis and specific medical counter measures. CWA/NA are extremely toxic agents which are subject to international regulations (The Geneva Protocol regarding the prohibition of chemical weapons). Taking into account the ban on human experimenting due to high toxicity, simulants of neurotoxic chemical agents are being used in laboratories. These are analogue chemical compounds which possess the same relevant physical, chemical and pharmacodynamical properties of the corresponding agents, but with lower intrinsical toxicity. The synthesis and toxicological characterization of these simulants of neurotoxic chemical agents allow the laboratory research of these extremely toxic compounds and the unfolding of risk-free antidote studies. This paper proposes the synthesis and toxicological testing of chemical compounds which simulate sarin (4-nitrophenyl isopropyl methylphosphonate - NIMP) and VX (4-nitrophenyl ethyl methylphosphonate - NEMP).

Keywords: NIMP, NEMP, neurotoxic chemical agents, acute toxicity, acetylcholinesterase (AchE)

High-risk neurotoxic chemical agents (sarin, soman, tabun, VX) are extremely toxic compounds which have structures of phosphoric acid esters [1-8]. Due to their extremely high toxicity, they were registered on the list of substances which must be destroyed according to the international regulations (Convention on the Prohibition of the Development, Production and Stockpiling of Chemical Weapons). Their production, stockpiling and use are monitored by international organizations (Organization for the Prohibition of Chemical Weapons - OPCW) [9-13]. Neurotoxic chemical agents have the property of irreversibly inhibiting the erythrocytary acetylcholinesterase (AChE) [14-18]. The coupling reaction of the organophosphorus compounds at the level of the active situs of the enzyme is represented in fig.1, 2.

The AChE inhibition leads to the accummulation of acetycholine (ACh) in the synaptic cleft and to the hyperstimulation of muscanic and nicotinic receptors [21, 22]. The clinical signs of mild intoxication are pupil constriction, sweating, muscle fasciculations, bradycardia and a decrease in blood pressure. At higher concentrations of the toxic agent, convulsions occur and, eventually, death through respiratory insufficiency due to the paralysis of respiratory centers. Cholinesterase inhibiting neurotoxic organophosphorus compounds possess, in their molecule, two organic groups, identical or different, and an active group, inorganic or organic, bound to a pentavalent phosphor atom. The central pentavalent phosphor atom can be bound to an oxygen or sulphur atom (phosphates, phosphonates, tionphosphates, tionphosphonates). In specialty scientific literature, potential simulators were presented for each class of warfare chemical neurotoxic agents [23-26]. The chemical compounds that were identified as possible simulants of neurotoxic agents, necessary in research activity regarding the optimization of specific treatment in the intoxication with neurotoxic organophosphoric compounds, are the non-volatile compounds: 4-nitrophenyl isopropyl methylphosphonate (NIMP) for sarin and 4-nitrophenyl ethyl methylphosphonate (NEMP) for VX. These maintain, within their molecule, the active group F-P=O which is responsible for the toxicological properties. In fig. 3, the chemical structure of neurotoxic agents and their corresponding simulants are being shown [4, 6, 14, 17, 24].
The laboratory use of neurotoxic chemical agents is restricted, as they are extremely toxic substances. The chemical compounds identified as simulators of these neurotoxic agents in studies regarding the optimization of specific treatment in the intoxication with neurotoxic organophosphoric compounds are two non-volatile compounds: a simulator of tabun, 4-nitrophenyl ethyl methylphosphonate (NEMP, a simulator for VX), and 4-nitrophenyl isopropyl methylphosphonate (NIMP) [23, 24]. Their synthesis was performed in the Chemical Analyses and Special Syntheses Laboratory, Section 1, of C.C.S.A.C.B.R.N.E.

General objectives
- the reduction in the risks the personnel which executes testing procedures for high-toxicity chemical compounds is subject to, through the synthesis and use of simulators of neurotoxic chemical agents in the laboratory.
- the optimization of medical countermeasures in the case of exposure to neurotoxic chemical agents through the extension of studies of antidotism with the condition of simulator use

Specific objectives
- The synthesis and chemical characterization of NIMP and NEMP simulators with purity of $\geq 90\%$
- The evaluation of the acute toxicity of NIMP and NEMP compared to their corresponding neurotoxic chemical agents
- The evaluation of their AChE inhibition properties compared to their analogues.

Experimental part

Materials and method
Methods of synthesis for simulators of neurotoxic agents Simulators are mostly esters (mono esters, symmetrical or asymmetrical diesters) of alkyl phosphonic acids, being obtained through a synthesis reaction which involves compounds called precursors. The esterification reactions are performed in the presence of a tertiary amine which has the role of blocking the formed hydrochloric acid and favoring the binding of the ester. The shifting of the equilibrium towards ester forming was performed through the use of an excess components (alcohol) and the unfolding of the reaction under inert gas pressure. The synthesis reactions were performed in two stages, at room temperature, within certain limits to avoid the decomposure of the product [5]. The syntheses were performed using methylphosphonic dichloride as a precursor, as it is a rather reactive substance and, thus, the esterification reaction can unfold without a catalyst. Using two different alcohols for esterification: ethanol and 2-propanol, the synthesis reaction of the two non-volatile compounds, NIMP and NEMP, identified as possible simulators of neurotoxic agents, are shown in fig. 5 and fig. 6 (after [27]):

Identification and characterization of simulator compounds were performed through gas-chromatography coupled with mass spectrometry (GC/MS). The chromatographic analysis performed with GC/MS Thermo Electron Corporation confirmed the structure of the analysed compounds.

RMN Characterization

Identification and characterization of simulator compounds were performed through gas-chromatography coupled with mass spectrometry (GC/MS). The chromatographic analysis performed with GC/MS Thermo Electron Corporation confirmed the structure of the analysed compounds.

The GC/MS mass spectrums of the two compounds, NIMP and NEMP, are shown in fig. 7 (a and b).

RIMP - Hydrogen spectrum

Spectrum $^1$H-RMN (CDCl$_3$, δ ppm, f Hz): 8.14(d, 9.3, 2H, H-2, H-6); 7.31(dd, 1.4, 9.3); 4.76(m, 1H, H-9); 1.60(d, 17.6, 3H, H-8); 1.28(d, 6.3, 3H, H-10); 1.19(d, 6.3, 3H, H-10') s = singlet, d = doublet, t = triplet, sx = sextet, m = multiplet

In the H spectrum, the para substitution through the 2 AB type systems for the 4 protons can be observed, paired as 2 equivalents, the most deshielded H$_2$, H$_6$ (higher δ) from 8.14 doublet with a coupling constant of 9.3 Hz, and the most shielded (orto in regards to oxygen) H$_3$, H$_5$ at 7.31 with the same coupling constant. The methyl group bound to a P is a doublet with ν = 18.0 ppm (coupled with phosphorus) and the isopropyl rest is confirmed by H-9
which is a multiplet at $\delta = 1.19$ ppm (which comes from a heptet) coupled with the two methyls and, also, a coupling with phosphorus appears, theoretically $7 \times 2 = 14$ lines. The methyl groups give 2 doublets with $\nu = 6.3$ Hz at 1.28 and 1.19 ppm (fig.8).
**NIMP - Carbon spectrum**

$^{13}$C-RMN spectrum (CDCl$_3$, δ ppm): 155.71(d, 7.7, C-4); 144.60(C-1); 125.62(d, 9.1, C-2, C-6); 121.12(d, 5.0, C-3, C-5); 72.36(d, 6.8, C-9); 23.98(d, C-8); 13.13(C-10); 11.81(C-10')

The carbon spectrum shows the aromatic nucleus which is para substituted and the coupling of carbon atoms with phosphorus. The most deshielded carbon atom is C-4 at δ = 155.71 ppm geminal with oxygen, after which is C-1 geminal with the nitro group at δ = 144.60 ppm. The tertiary equivalent carbon atoms C-2 and C-6 appear at δ = 125.62 ppm and C-3, C-5, also equivalents, appear at δ = 121.12 ppm. The remaining alkyl appear as CH (isopropyl) at δ = 72.36 ppm, CH$_3$-P group at δ = 23.98 ppm and the two methyl groups of isopropyl at 13.13 and 11.81 ppm (Fig. 9).

**4-Nitrophenyl 2-ethylmethylphosphonate - NEMP**: (simulator of VX)

RMN characterization

NEMP – Hydrogen spectrum $^1$H-RMN (CDCl$_3$, δ ppm, ν Hz): 8.17(d, 9.3, 2H, H-2, H-6); 7.33(dd, 1.1, 9.3, 2H, H-3, H-5); 4.15(m, 2H, H-9); 1.63(d, 18.0, 3H, H-8); 1.27(t, 7.1, 3H, H-10) Para substitution shows for the 4 aromatic protons two AB systems with a coupling of 9.3 Hz. H-2 and H-6 equivalents protons appear at δ = 8.17 ppm, more deshielded due to the NO$_2$ group, and the most shielded are H-3, H-5 (due to the oxygen) at δ = 7.33 ppm. The remaining ethyl is confirmed by the multiplet at 4.15 ppm (Theoretically a dedoubled coupling quartet with the phosphorus), for the methylen group H-9, and the ethyl group through the triplet at 1.27 ppm with the coupling constant of 7.1 Hz. The methyl group bound to phosphorus is confirmed by the doublet of 18.0 Hz at 1.63 ppm, due to coupling with phosphorus (fig. 10).
The pharmacotoxicological characterization of the tested compounds
The in vivo testing of the acute toxicity of the two compounds, NIMP and NEMP (DL_{50})

Tested substances:
- 4 nitrophenyl isopropyl methylphosphonate (NIMP) synthethised and characterized in the Chemical Analyses and Special Syntheses Laboratory, Section 1, of C.C.S.A.C.B.R.N.E.
- 4 nitrophenyl ethyl methylphosphonate (NEMP) synthethised and characterized in the Chemical Analyses and Special Syntheses Laboratory, Section 1, of C.C.S.A.C.B.R.N.E.

The preparation of solutions:
- NIMP: 0.033 mL are dissolved in 5 mL DMSO. From this solution, 1 ml is dissolved in 19 ml distilled water in a graded 20 ml bottle leading to a working solution of 0.38 mg/mL.
- NEMP: 0.040 mL are dissolved in 5 mL DMSO. From this solution, 1 mL is dissolved in 19 mL distilled water in a graded 20 mL bottle leading to a working solution of 0.40 mg/mL.

Biological material
10 groups of 10 animals were used in the experiment (male Wistar rats) weighing on average 200-250g, fed a daily constant portion of standard food, with free access to water, kept in optimal temperature, humidity, lighting and away from contact with pesticides.

Experimental protocol
The following pharmacodynamic parameters were determined: DL_{50}, and the inhibition degree of erythrocytary AChE and plasmatic BuChE for the two selected surrogate compounds, as well as the protection index after the administration of a fixed antidote ATOX combination ATOX. Each intoxicated and treated group was considered a control group for itself. The NIMP and NEMP doses administered experimentally were calculated in geometrical progression utilizing the literature references.

Group 1 - This group was intoxicated with a dose of NIMP of 0.59 mg/kg i.p. coded as the high dose. The mortality and intoxication signs were examined. Every 60 minutes from intoxication, 0.2 mL of blood were sampled from the same animals on an anticoagulant to measure the inhibition of erythrocytary Acetylcholinesterase.

Group 2 - This group was intoxicated with a dose of NIMP of 0.47 mg/kg i.p. coded as the moderate dose. The mortality and intoxication signs were examined. Every 60 minutes from intoxication, 0.2 mL of blood were sampled from the same animals on an anticoagulant to measure the inhibition of erythrocytary Acetylcholinesterase.

The method of measuring acetylcholinesterase levels: was performed using the AChE Elisa rat kit for determining acetylcholinesterase in rats. Initially, at the time considered

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NEMP - Carbon spectrum

$^1$C-RMN spectrum (CDCl$_3$, 6 ppm): 155.65(d, 8.0, C-4); 144.70(C-1); 125.82(d, 12.8, C2-C6); 121.70(4.9, C3-C5); 63.11(C-9); 16.40(d, 6.0, C-8); 12.70(C-10) (fig. 11).
To, 1 mL of blood was sampled on citrate to determined normal values of acetylcholinesterase through the Elisa rat kit micromethod. The blood samples were processed according to the working protocol of AchE Elisa rat kit micromethod. For the statistical analysis, the T Student test, ANOVA and TURKEY were used.

Results and discussions

Determining the DL₅₀ interval for the NIMP compound

The steps for analysing the results corresponding to the determining of the DL₅₀ interval for the NIMP compound are shown in table 1 and fig. 12.

The square value of the correlation coefficient is 0.991, and the logarithm values of the doses administered to groups 1,2,3, being approximately on the regression line, show the experimental results are correct and that the DL₅₀ interval for the NIMP compounds is between 0.32 - 0.59 mg/kg i.p. DL₅₀ resulted from calculation (represented by the value of x if, in the regression line, the value of y is 5), is 0.45 ± 0.002mg/kg i.p.

Determining the DL₅₀ interval for the NEMP compound

The steps for analysing the results corresponding to the determining of the DL₅₀ interval for the NEMP compound are shown in table 2 and fig. 13.

Doses of NIMP determine an inhibition of AchE of 95.72%, 87.38 % and 53.15% (fig. 14).

<table>
<thead>
<tr>
<th>No.</th>
<th>Gr. no.</th>
<th>NIMP dose mg/kg</th>
<th>Dose logarithm</th>
<th>Percentile mortality probit</th>
<th>Percentile mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gr. 1</td>
<td>0.59</td>
<td>-0.23</td>
<td>5.81</td>
<td>80%</td>
</tr>
<tr>
<td>2</td>
<td>Gr. 2</td>
<td>0.47</td>
<td>-0.33</td>
<td>4.86</td>
<td>60%</td>
</tr>
<tr>
<td>3</td>
<td>Gr. 3</td>
<td>0.32</td>
<td>-0.42</td>
<td>4.24</td>
<td>20%</td>
</tr>
</tbody>
</table>

Legend: X axis - logarithm values of doses administered to the 3 groups in the experiment; Y axis - values of corresponding probits of percentile mortality for each group.

<table>
<thead>
<tr>
<th>No.</th>
<th>Gr. no.</th>
<th>NEMP dose mg/kg</th>
<th>Dose logarithm</th>
<th>Percentile mortality probit</th>
<th>Percentile mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gr. 4</td>
<td>0.64</td>
<td>-0.19</td>
<td>5.94</td>
<td>70%</td>
</tr>
<tr>
<td>2</td>
<td>Gr. 5</td>
<td>0.51</td>
<td>-0.29</td>
<td>5</td>
<td>50%</td>
</tr>
<tr>
<td>3</td>
<td>Gr. 6</td>
<td>0.4</td>
<td>-0.39</td>
<td>4.10</td>
<td>10%</td>
</tr>
</tbody>
</table>

Legend: X axis - logarithm values of doses administered to the 3 groups in the experiment; Y axis - values of corresponding probits of percentile mortality for each group.

The square value of the correlation coefficient is 0.998, and the logarithm values of the doses administered to groups 4, 5, 6, being approximately on the regression line, show the experimental results are correct and that the DL₅₀ interval for the NEMP compounds is between 0.4 - 0.64 mg/kg i.p. and DL 50 (represented by the value of x if, in the regression line, the value of y is 5) is 0.51 ± 0.003mg/kg i.p.

Table 1

<table>
<thead>
<tr>
<th>No.</th>
<th>Gr. no.</th>
<th>NIMP dose mg/kg</th>
<th>Dose logarithm</th>
<th>Percentile mortality probit</th>
<th>Percentile mortality</th>
</tr>
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<tr>
<td>1</td>
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<td>0.59</td>
<td>-0.23</td>
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<td>80%</td>
</tr>
<tr>
<td>2</td>
<td>Gr. 2</td>
<td>0.47</td>
<td>-0.33</td>
<td>4.86</td>
<td>60%</td>
</tr>
<tr>
<td>3</td>
<td>Gr. 3</td>
<td>0.32</td>
<td>-0.42</td>
<td>4.24</td>
<td>20%</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>No.</th>
<th>Group</th>
<th>Mean value of AchE (U/L) before intoxication</th>
<th>Dose of NIMP (mg/kg) administered i.p</th>
<th>Mean value of AchE activity (nU/L) 60 minutes from intoxication</th>
<th>P probability associated with T Student test</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gr. 1</td>
<td>0.59</td>
<td>0.59</td>
<td>0.02</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Gr. 2</td>
<td>0.47</td>
<td>0.56</td>
<td>0.04</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Gr. 3</td>
<td>0.31</td>
<td>2.36</td>
<td>0.005</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Gr. 7</td>
<td>4.44</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Table 3

Fig. 12. Calculating DL₅₀. Representation of the regression line and of correlation coefficient.

Fig. 13. Representation of the regression line and of correlation coefficient.

Table 3

THE EVALUATION OF ACH ENHANCEMENT ACTIVITY FOR NIMP
Doses of administered NEMP determine an AChE inhibition of 88.73%, 77.92, and 55.9% (fig. 15). Statistical Anova and Turkey analyses of the experimental results complete the aspects shown above, revealing statistically significant differences (p < 0.05) between mean values of AChE inhibition, which were determined by the administered doses of the two studied compounds.

Table 4
THE EVALUATION OF ACHE INHIBITION ACTIVITY FOR NIMP

<table>
<thead>
<tr>
<th>No.</th>
<th>Group</th>
<th>Mean value of AChE (U/L) before intoxication</th>
<th>Dose of NEMP (mg/kg) administered i.p.</th>
<th>Mean value of AChE activity (mU/L) 60 minutes from intoxication</th>
<th>P probability associated with T Student test</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Group 4</td>
<td>0.64</td>
<td>0.5</td>
<td>0.003</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Group 5</td>
<td>0.51</td>
<td>0.98</td>
<td>0.004</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Group 6</td>
<td>0.40</td>
<td>2.46</td>
<td>0.005</td>
<td>0.005</td>
<td></td>
</tr>
</tbody>
</table>

Doses of administered NEMP determine an AChE inhibition of 88.73%, 77.92, and 55.9% (fig. 15). Statistical Anova and Turkey analyses of the experimental results complete the aspects shown above, revealing statistically significant differences (p < 0.05) between mean values of AChE inhibition, which were determined by the administered doses of the two studied compounds.

NIMP

\[
\begin{align*}
\text{NIMP} & \\
\text{ace} & \text{lot} \\
1 & 4.20 \text{ normal} \\
2 & 3.60 \text{ normal} \\
3 & 3.30 \text{ normal} \\
4 & 5.30 \text{ normal} \\
5 & 4.80 \text{ normal} \\
6 & 0.20 \text{ high dose} \\
7 & 0.18 \text{ high dose} \\
8 & 0.63 \text{ moderate dose} \\
9 & 0.68 \text{ moderate dose} \\
10 & 0.40 \text{ moderate dose} \\
11 & 0.33 \text{ moderate dose} \\
\end{align*}
\]

Tukey multiple comparisons of means 95% family-wise confidence level

\[
\begin{align*}
\text{diff} & \text{ lwr} \text{ upr} \text{ p adj} \\
\text{normal-h. dose} & 0.37 \text{ -1.088} 1.823 0.7562 \\
\text{normal-l. dose} & 4.05 \text{ 2.641 5.459 0.0001} \\
\text{normal m. dose} & 3.68 \text{ 2.550 4.810 0.0000} \\
\end{align*}
\]

NEMP

\[
\begin{align*}
\text{NEMP} & \\
\text{ace} & \text{lot} \\
1 & 4.20 \text{ normal} \\
2 & 3.60 \text{ normal} \\
3 & 3.30 \text{ normal} \\
4 & 5.30 \text{ normal} \\
5 & 4.80 \text{ normal} \\
6 & 0.20 \text{ high dose} \\
7 & 0.18 \text{ high dose} \\
8 & 0.63 \text{ moderate dose} \\
9 & 0.68 \text{ moderate dose} \\
10 & 0.40 \text{ moderate dose} \\
11 & 0.33 \text{ moderate dose} \\
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\end{align*}
\]
Tukey multiple comparisons of means 95% family-wise confidence level

diff  lwr  upr  p adj
m. dose  - h. dose  0.4973  -0.6827  1.677  0.5118
normal-h. dose  3.9100  2.7674  5.053  0.0000
normal-m. dose  3.4127  2.4342  4.391  0.0000

The analysis of percentile results showed that the two studied compounds have comparable toxicological AChE inhibition effects, toxicity being higher for NIMP for the studied dosing intervals.

- The syntheses' main objective was obtaining non-volatile chemical compounds that can be utilized as neurotoxic agents simulators: in this case, phosphorylate cholinesterase with the same radicals as the respective neurotoxic agents, thus becoming relevant for the studying of cholinesterase reactivators. The results have confirmed that the proposed standards for the two compounds, NIMP and NEMP, were reached with a purity higher than 90%.

**Simulator compound NIMP:**
- has a DL 50 of 0.45 ± 0.002mg/kg i.p in the interval of 0.32- 0.59 mg/kg i.p.
- administering three doses in logarithmic progression in the above interval shows, dose-correlated, an effect of erythrocytary AChE inhibition of 53 to 95%, values that are comparable to those determined by the reference compound.

**Simulator compound NEMP:**
- has a DL 50 of 0.51 ± 0.003mg/kg i.p in the interval of 0.4 - 0.64 mg/kg i.p.
- administering three doses in logarithmic progression in the above interval shows, dose-correlated, an effect of erythrocytary AChE inhibition of 56 to 88%, values that are comparable to those determined by the reference compound.

- The two studied compounds present erythrocytary AChE inhibition properties comparable to the reference substances, which qualifies them for antidotism studies.
- The comparative analysis of the toxicological properties showed the fact that the acute intrinsic toxicity (DL₅₀) of the studied compounds is smaller than that of the anologue neurotoxic chemical agents (sarin and VX-NATO standards as references) [28, 29].

**Conclusions**

The syntheses' main objective was obtaining non-volatile chemical compounds that can be utilized as neurotoxic agents simulators: in this case, phosphorylate cholinesterase with the same radicals as the respective neurotoxic agents, thus becoming relevant for the studying of cholinesterase reactivators. The results have confirmed that the proposed standards for the two compounds, NIMP and NEMP, were reached with a purity higher than 90%.

The anticholinesterase activity of these simulators of neurotoxic agents is sufficient to mimic the exposure to them, all clinical signs typical of this intoxication being present. As is the case with acetylcholinesterase aging related to sarin and VX, the action of these surrogates shows a slow rate of aging, allowing for a window of therapeutic opportunity for the reactivating oximes. The potency and enzymatic aging rate induced by these simulators make them especially useful in the unfolding of antidotism studies.

Thus, we can conclude that the two studied compounds, NIMP and NEMP have AChE inhibition effects that are similar to the references and, due to their reduced intrinsic toxicity, can be adequate as simulators for antidotism studies in the laboratory.

**ABBREVIATIONS:**
- **NIMP 4-nitrophenyl isopropyl methylphosphonate;**
- **NEMP 4-nitrophenyl ethyl methylphosphonate;**
- **GC-MS gas chromatography - mass spectrometry;**
- **RMN nuclear magnetic resonance;**
- **CWA/NA neurotoxic agents;**
- **AChE acetylcholinesterase;**
- **CCSMM Medical-Military Scientific Research Center;**
- **C.C.S.A.C.B.R.N.E. Scientific Research Center for CBRN Defense and Ecology**

**References**