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, simulators 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 simulators 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 organophosphoric compounds at the level of the active situs of the enzyme is represented in fig.1, 2.

The AChE inhibition leads to the acummulation of acetycholine (ACh) in the synaptic cleft and to the hyperstimulation of muscarinic 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 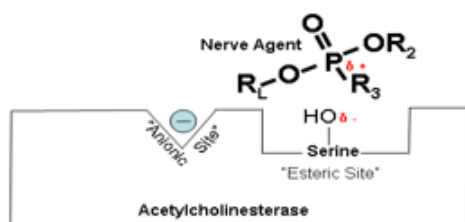organophosphoric compounds possess, in their molecule, two organic groups, identical or different, and and active group, inorganic or organic, bound to a pentavalent phosphor atom. The central pentavalent phosphor atom can be bound to an oxygen or sulphur atom (phosphates, phosphonates, tionphosphates, 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 simulators of neurotoxic agents, necessary in research activity regarding the optimization of specific treatment in the intoxication with neurotoxic organophosphoric compounds, are the non-volatile compunds: 4-nitrophenyl isopropylic methylphosphonate (NIMP) for sarin and 4-nitrophenyl ethyl methyl-phosphonate (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 simulators are being shown [4, 6, 14, 17, 24].
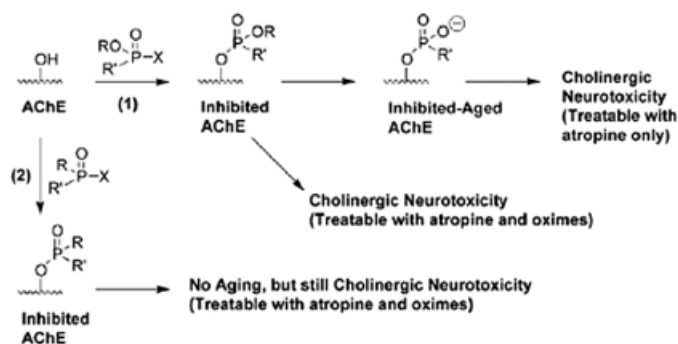

Fig. 1. The coupling mechanism of the organophosphoric compound at the level of the active situs of AChE (diagram modified from [19])

Fig. 2. Interaction between AchE and organophosphoric compound (after [20])
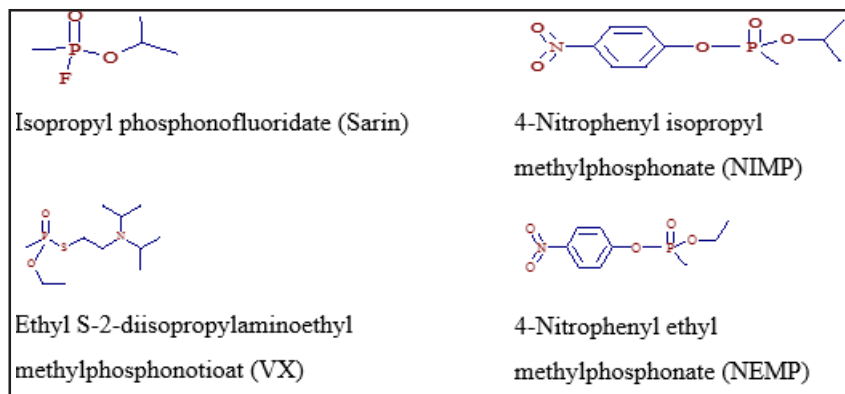
Fig. 3. Chemical structure of neurotoxic agents and their corresponding simulators

Isopropyl phosphonofluoridate (Sarin)

4-Nitrophenyl isopropyl methylphosphonate (NIMP)

Ethyl S-2-diisopropylaminoethyl methylphosphonotioat (VX)

4-Nitrophenyl ethyl methylphosphonate (NEMP)

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, the mass temperature being maintained 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, in an alkylphosphonic dichloride/alcohol molar ratio of 1/2, the

nitrophenyl ester omologues identified as simulators of neurotoxic chemical agents were obtained. The final reaction product is an oily liquid which is homogenous and of brown color.

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]):
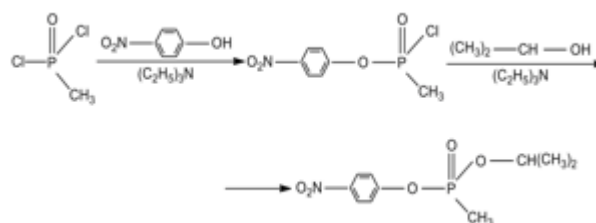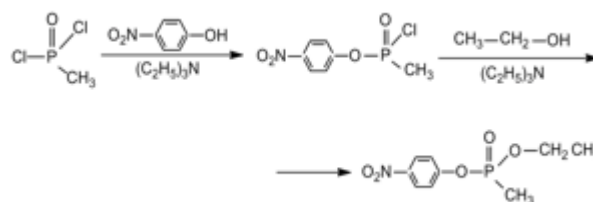

Fig. 5. Synthesis reaction of NIMP

Fig. 6. Synthesis reaction of NEMP

Identification and characterization of simulator compounds were performed through gas-chromatography coupled with mass spectrometry (GC/MS). The chromatographic analysis performed with GC/MS DSQ II 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).

RMN Characterization
The purity of NIMP and NEMP compounds, determined with RMN 1H, was of 90% and 94%.

NIMP - Hydrogen spectrum
Spectrum ^{1}H-RMN (CDCl$_3$, δ ppm, í 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 dublet 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
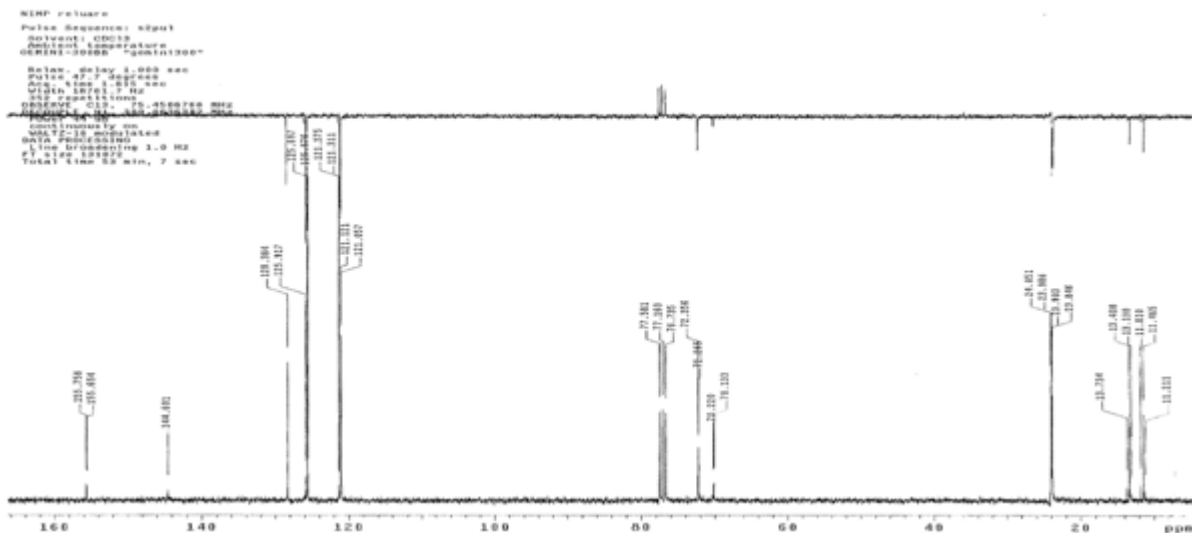
which is a multiplet at δ=1.19 ppm (which comes from a heptet) coupled with the two methyls and, also, a coupling with phosphorus appears, theoretically 7x2=14 lines. The methyl groups give 2 doublets with ν=6.3 Hz at 1.28 and 1.19 ppm (fig.8).

Fig. 8. NIMP - Hydrogen spectrum

Fig. 9. NIMP - Carbon spectrum

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 ä=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 11.81ppm (Fig. 9).

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

NEMP – Hydrogen spectrum ^1H-*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)
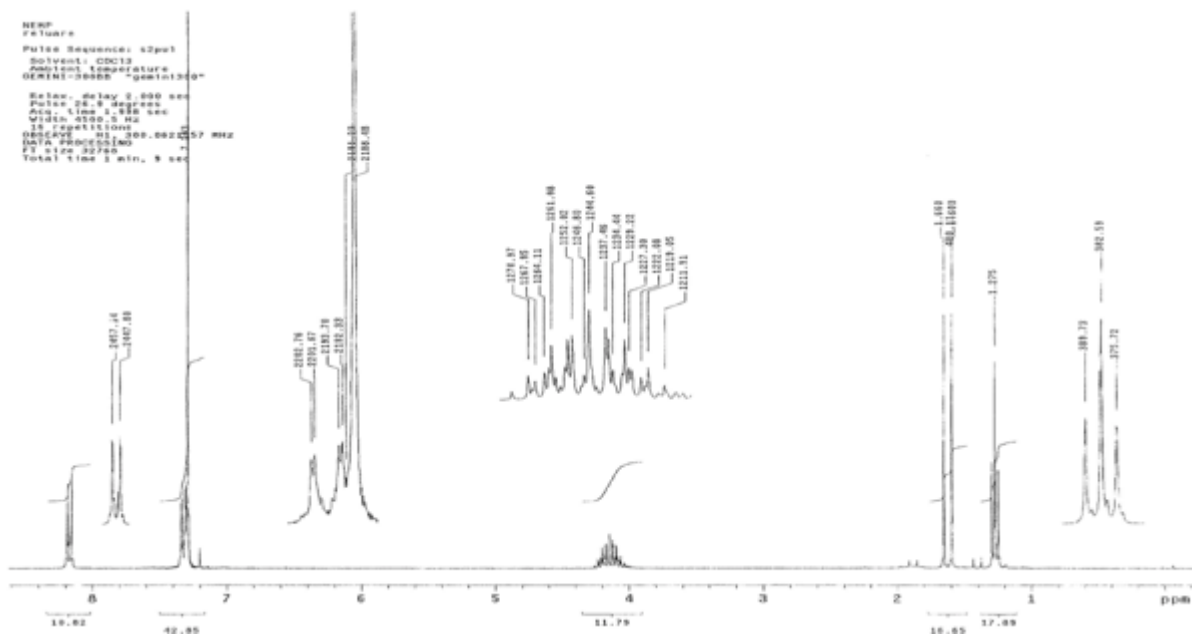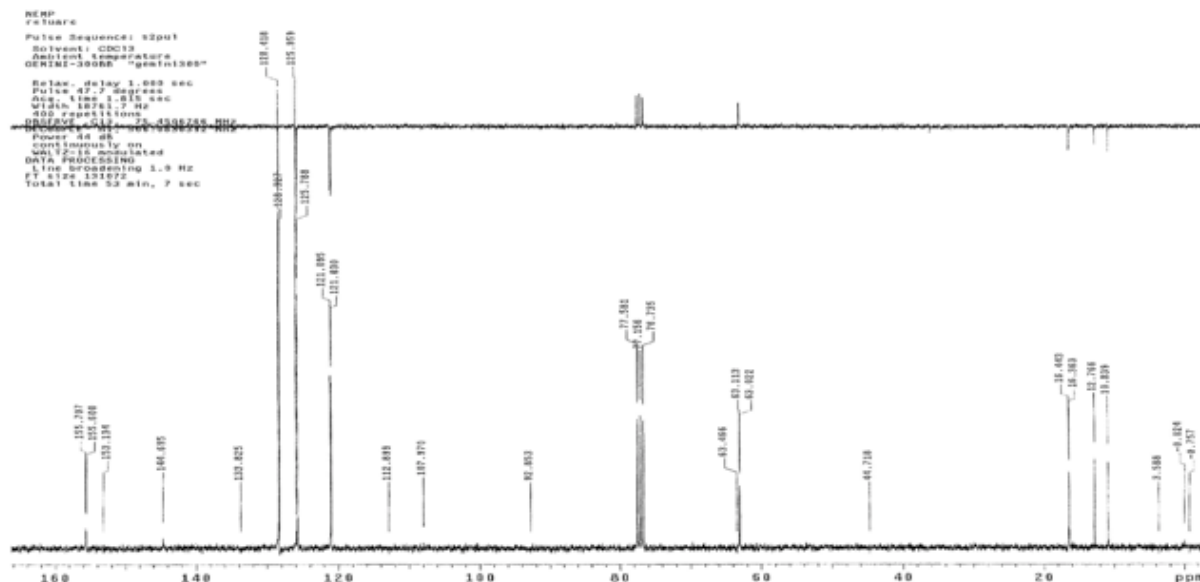

Fig. 10. NEMP - Hydrogen spectrum

Fig. 11. NEMP Carbon spectrum

NEMP- Carbon spectrum

^{13}C-RMN spectrum (CDCI$_3$, δ 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).

The pharmacotoxicological characterization of the tested compounds
The in vivo testing of the acute toxicity of the two compunds, 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 erythrocitary 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 erythrocitary Acetycholinesterase inhibition.

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 erythrocitary Acetycholinesterase inhibition.

Group 3 - was intoxicated with a dose of *NIMP of 0.38 mg/kg i.p.* coded as the low 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 erythrocitary Acetycholinesterase inhibition.

Group 4 - was intoxicated with a dose of *NEMP of 0.64 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 erythrocitary Acetycholinesterase inhibition.

Group 5 - was intoxicated with a dose of *NEMP of 0.51 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 erythrocitary Acetycholinesterase inhibition.

Group 6 - 0.2 mL of blood was sampled through cardiac punction on an anticoagulant for the spectrophotometric measuring of normal values of erythrocitary acetyl-cholinesterase; Eventually, they were intoxicated with a dose of *NEMP of 0.40 mg/kg* i.p. coded as the low 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 erythrocitary Acetycholinesterase inhibition.

Group 7 (control group) was administered 1.5 mL of physiological serum and s0.2 mL of blood was sampled through cardiac punction on an anticoagulant for the spectrophotometric measuring of normal values of erythrocitary 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

T0, 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 DL$_{50}$ interval for the NIMPcompound

The steps for analysing the results corresponding to the determining of the DL$_{50}$ 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 logarhythm 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 DL$_{50}$ interval for the NIMP compounds is between 0.32 - 0.59 mg/kg i.p. DL$_{50}$ 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 DL$_{50}$ interval for the NEMP compound

The steps for analysing the results corresponding to the determining of the DL$_{50}$ 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).

No.	Gr. no.	NIMP dose mg/kg	Dose logarhythm	Percentile mortality probit	Percentile mortality
1	Gr. 1	0.59	-0.23	5.81	80%
2	Gr. 2	0.47	-0.33	4.86	60%
3	Gr. 3	0.32	-0.42	4.24	20%

Table 1
STEPS OF STATISTICAL ANALYSIS AND OF CALCULATING OF DL 50 INTERVAL

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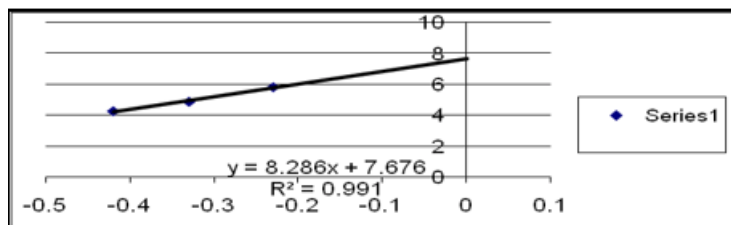*Y axis - values of corresponding probits of percentile mortality for each group*

Fig. 12. Calculating DL$_{50}$ Representation of the regression line and of correlation coefficient

No.	Gr. no.	NEMP dose mg/kg	Dose logarhythm	Percentile mortality probit	Percentile mortality
1	Gr. 4	0.64	-0.19	5.94	70%
2	Gr. 5	0.51	-0.29	5	50%
3	Gr. 6	0.4	-0.39	4.16	10%

Table 2
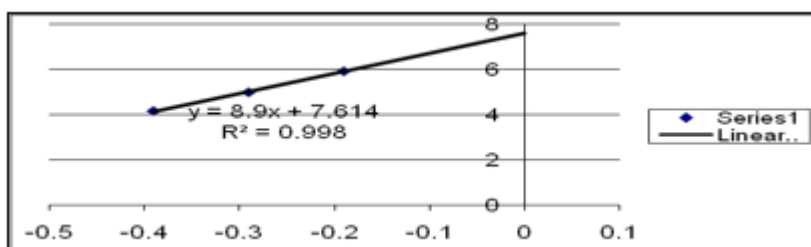STEPS OF STATISTICAL ANALYSIS AND OF CALCULATING OF DL 50 INTERVAL

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

Legend: X axis- logarhythm 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 logarhythm 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 DL$_{50}$ 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 3
THE EVALUATION OF ACHE INHIBITION ACTIVITY FOR NIMP

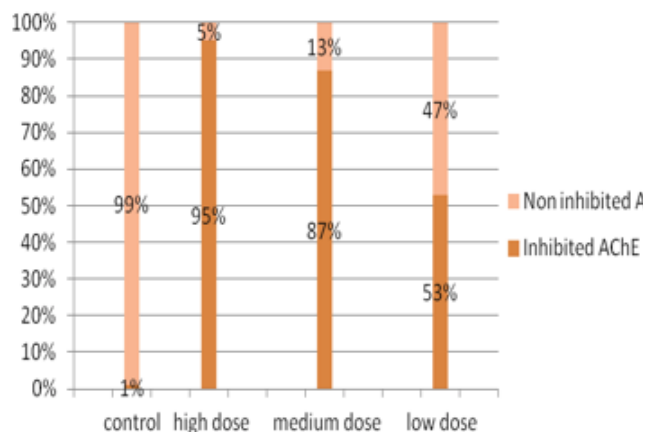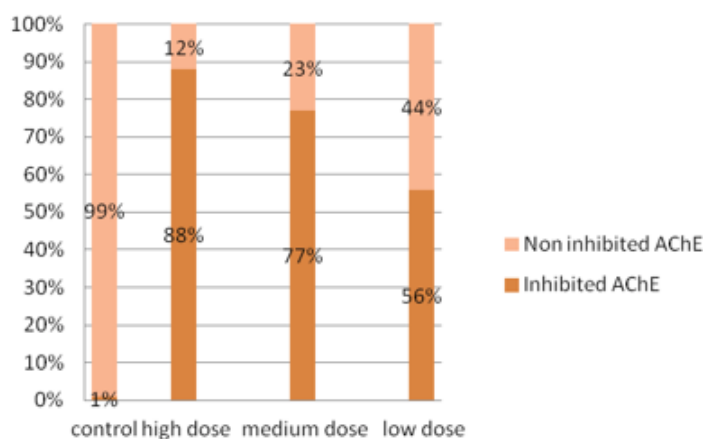| No.	Group	Mean value of AchE (U/L) before intoxication	Dose of NIMP (mg/kg) administered i.p	Mean value of AChE activity (mU/L) 60 minutes from intoxication	P probability associated with T Student test	Observations
1	Gr. 1		0.59	0.19	0.003	P< 0,05 shows the initial mean values of AchE differ in a statistically significant way from those after intoxication
2	Gr. 2		0.47	0.56	0.004	
3	Gr. 3		0.31	2.36	0.005	
4	Gr. 7	4.44	-	-		

Fig. 14. AchE inhibition determined by NIMP

Fig.15. AChE inhibition determined by NEMP

Table 4
THE EVALUATION OF ACHE INHIBITION ACTIVITY FOR NIMP

No.	Group	Mean value of AchE (U/L) before intoxication	Dose of NEMP (mg/kg) administered i.p.	Mean value of AchE activity (mU/L) 60 minutes from intoxication	P probability associated with T Student test	Observations
1	Group 4		0.64	0.5	0.003	P< 0,05 shows the initial mean values of AchE differ in a statistically significant way from those after intoxication
2	Group 5		0.51	0.98	0.004	
3	Group 6		0.40	2.46	0.005	
4	Group 7	4.44	-	-	-	

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

```
  ache    lot
1 4.20  normal
2 3.60  normal
3 3.30  normal
4 5.30  normal
5 4.80  normal
6 0.20  high dose
7 0.18  high dose
8 0.63  moderate dose
9 0.68  moderate dose
10 0.40  moderate dose
11 0.53  moderate dose
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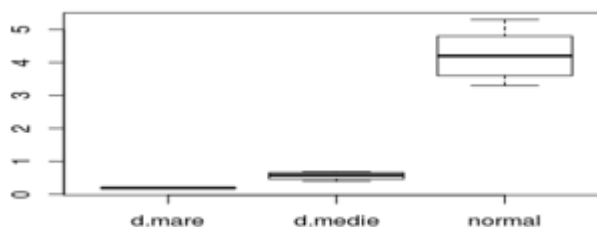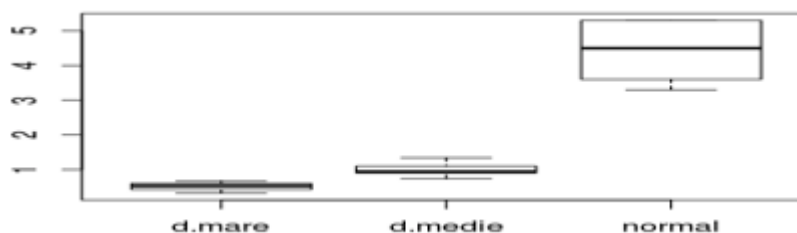

Tukey multiple comparisons of means 95% family-wise confidence level

```
              diff    lwr    upr   p adj
m. dose-h. dose 0.37 -1.088 1.828 0.7562
normal-h. dose 4.05  2.641 5.459 0.0001
normal-m. dose 3.68  2.550 4.810 0.0000
```

NEMP

```
   ache    lot
1  4.20  normal
2  3.60  normal
3  3.30  normal
4  5.30  normal
5  4.80  normal
6  5.30  normal
7  0.53  high dose
8  0.67  high dose
9  0.32  high dose
10 0.74  moderate dose
11 0.94  moderate dose
12 0.90  moderate dose
13 1.34  moderate dose
14 1.10  moderate dose
```

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, to 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 logarhythmic 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 logarhythmic 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 (DL_{50}) of the studied compounds is smaller than that of the analogue 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, to 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 tipical of this intoxication being present. As is the case with acetylcholinesterase aging related to sarin and XV, 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.

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