# ФАРМАКОЛОГИЯ, ТОКСИКОЛОГИЯ И ФАРМАЦЕВТИЧЕСКИЕ НАУКИ

## EVALUATION OF ANTICONVULSANT ACTION OF PROPOXAZEPAM ON PENTYLENETETRAZOLE-KINDLING MODEL OF SEIZURE IN MICE

***Golovenko Nikolay Yakovlevich,***
*Doctor of science (Biology), professor,*
*Leading Researcher, V. Bogatsky Physico-Chemical Institute of NAS of Ukraine, Odessa.,*
***Larionov Vitalii Borisovich,***
*Doctor of science (Biology), Senior Researcher,*
*V. Bogatsky Physico-Chemical Institute of NAS of Ukraine, Odessa.,*
***Reder Anatolii Semenovich***
*PhD (Chemistry), Senior Researcher,*
*V. Bogatsky Physico-Chemical Institute of NAS of Ukraine, Odessa.,*
***Andronati Sergiy Andriyovich,***
*Doctor of science (Chemstry), professor,*
*Head of department of Medicinal Chemistry, V. Bogatsky Physico-Chemical Institute of NAS of Ukraine, Odessa.,*
***Valivodz Irina Petrivna,***
*Junior Research Fellow,*
*V. Bogatsky Physico-Chemical Institute of NAS of Ukraine, Odessa.,*

**Abstract**

Kindling model of epilepsy on mice was induced by subcutaneous pentylenetetrazol (PTZ) injections (30 mg/kg) each second-third day during 29 days. Experimental animals were treated with propoxazepam (0,2; 0,6; 1,0 and 2.0 mg/kg, intraperitonealy) 30 min prior to convulsive agent injection. Quantities of different seizures types (in accordance to standard scale) were counted during 1 hour after PTZ injection. The highest quantity of minimal severity seizures (1 point) was registered at animals of control group from $3^{th}$ to $8^{th}$ and after $8^{th}$ PTZ injections. Propoxazepam administration did not lead to reliable changes of seizures manifestations, but constrained second type seizures development, reducing their quantity. The statistically significant redistribution in seizures quantity of second and fourth severity types with simultaneous increasing of higher severity seizures was noted. At the same time seizures of fourth severity type were diminished. For animals of control and experimental groups the reducing of first and second severity seizures types latent time was noted. With propoxazepam administration reducing of higher severity seizures types ($3^{th}$ and $4^{nd}$ type) was in accordance with reducing of the latency time for seizures of $1^{th}$ and $2^{nd}$ types.

**Keywords:** propoxazepam, pentylenetetrazol kindling epilepsy, antiepileptic effects, components of seizure.

### INTRODUCTION

Epilepsy refers to chronic polyethyologic diseases of the brain characterized by recurrent seizures that occur as a result of excessive neuronal discharges and accompanied by various clinical and paraclinical symptoms. Anticonvulsant therapy remains the basis for treating patients with epilepsy, which involves inhibition or a significant reduction in the number of attacks. Currently, the term "antiepileptic" is synonymous with "anticonvulsant agents" as they all selectively suppress seizure and their use is determined predominantly by the nature of paroxysmal manifestations or its equivalents [1]. Depending on the clinical manifestations of epilepsy, different anticonvulsants can be prescribed. Often, for the treatment of epilepsy, combined use of several medicines is rational (simultaneously or sequentially). Therefore, the success of the treatment of epilepsy is on the way to finding new anticonvulsants, which would have had an effect on different pathogenesis links in the formation of all variability of seizure states [2].

A novel 3-substituted 1,4-benzodiazepine, 7-bromo-5-(*o*-chlorophenyl)-3-propoxy-1,2-dihydro-3H-1,4-benzodiazepin-2-one (named propoxazepam), has been found to have a potent anticonvulsant effect [3]. In models of chemically induced seizures we determined [4] the average effective doses ($ED_{50}$) of propoxazepam as an antagonist to picrotoxin (1.67 ± 0.09 mg/kg), pentylenetetrazol (0.9 ± 0.04 mg/kg), and strychnine (4.24 ± 0.47 mg/kg), which reflect the high activity level of the substance. Propoxazepam had shown high activity on the model of GABA-deficient thiosemicarbazide-induced convulsions [5]. Propoxazepam mean effective doses in acute (3 hours) and remote (24 hours) periods of supervising have no statistically significant difference.

In this study, the anticonvulsant effect of propoxazepam was determined in pentylenetetrazol-induced kindling model of seizures in mice for further determination of antiepilectic properties.

### MATERIAL AND METHODS

Male mice (18-26 g), obtained from Institute of Pharmacology and toxicology NAMS of Ukraine housed at the local animal department, were used. The animals were exposed to a 12 h light-dark cycle and were provided with food and water *ad libitum*. All experiments were conducted during the light part of the day (9.00-14.00). The experiments were carried out according to the recommendations of the Committee for Research and Ethical Issues

of the IASP (1983) and were approved by the regional ethical committee for animal research. All manipulations were made to minimize animal suffering and to reduce the number of animals used.

The test compounds were suspended in tween 80 (1%) emulsion, and the control animals received corresponding amount of vehicle (1% tween 80).

Propoxazepam was synthesized according to the method described in [3]. The structure of the substance was determined and approved by a complex of physicochemical methods (IR and mass spectroscopy, as well as X-ray diffraction analysis). Chemical purity was confirmed by elemental analysis (99%). pentylenetetrazol (PTZ) was obtained from Sigma, USA.

**Induction of kindling by pentylenetetrazol (PTZ) injections**

All the animals except the normal control were injected with a subconvulsive dose of PTZ (30 mg/kg) on every alternate day to induce kindling. Propoxazepam was injected intraperitoneally 30 mins prior to each PTZ injection for 30 days. Prolonged administration of PTZ in subdoses (25-35 mg/kg) is a characteristic condition for kindling. In this study, the chemoconvulsant administration scheme was chosen in 2-3 days, which combines the reduction of traumatic in animals (due to parenteral administration) and the ability to achieve the necessary kindling indicators.

As recorded indicators of propoxazepam activity, the number of convulsive episodes of varying severity and the time of their development from the chemoconvulsant administration time were used [4]. Seizure attacks were grouped according to severity depending on external signs according to the standard 5-point scale:

O points: No response

1 point: Ear and facial twitching

2 points: myoclonic body jerks

3 points: clonic forelimb convulsions

4 points: generalized clonic convulsions, turning onto one side position

5 points: generalized clonic-tonic convulsions (or death within 30 minutes)

For studying on the model of the kindling-epilepsy were taken doses of 1.0 mg/kg (close to $ED_{50}$) and doses with an average step of 0.33 on a logarithmic scale - twice (2.0 mg/kg) and doses within the range one (0.6 mg/kg) and two (0.2 mg/kg) standard deviations from the average dose, corresponding to an effect of ~ 22% and ~ 7% of animals according to the dose-effect curve. The data were presented in the form of "mean ± standard deviation in the data set".

The relative contribution of convulsive attacks of varying degrees of severity to the total amount of seizure readiness in each group was determined as the normalized ratio of the sum of seizure attacks of each group to the total number of events in the group:

$$I_i = \frac{N_i}{N} 100\%$$

where $I_i$ - contribution (in %) of seizure attacks of groups of severity i (1-5), $N_i$ - number of convulsive attacks of the corresponding group, N - total number of seizure attacks in the group of animals.

The integral characteristic of the development of the kindling state in each experimental animal group was carried out using the index of convulsions (kindling):

$$I = \frac{A + 2B + 3C + 4D + 5E}{A + B + C + D + E}$$

Where A, B, C, D and E are the number of convulsive attacks of varying severity for each animal, respectively.

**Statistical analysis**

A preliminary assessment of the nature of the distribution of each type of data for compliance with the normal distribution law was carried out using asymmetry and excessive indices. If the indicators of these values did not exceed the value of the corresponding errors by more than 2 times, the Student's criteria was used as the characteristic of the difference, and the data was represented as "mean ± standard deviation in the data set". In the case of a significant deviation of the distribution from the Gauss one the assessment of the probability of the difference between the groups performed using methods of nonparametric statistics (Mann-Whitney U-test) [5].

**RESULTS**

Propoxazepam has previously been reported to have anticonvulsant effects for acute seizures in mice [6-8]. In the present work we report anticonvulsant effects of propoxazepam in a chronic model (PTZ-induced kindling,). PTZ-kindling is a well-studied animal model which simulates clinical epilepsy. It is a commonly preferred behavioral approach used for chemical kindling to study brain excitability [9]. Absence, myoclonic and generalized tonic-clonic seizures are induced by PTZ administration.

The analysis of anticonvulsant activity of propoxazepam on the model of PTZ induced kindling in mice (equivalent to absence form of epilepsy) was performed, and the dynamics of convulsions redistribution of various severity degrees was characterized. PTZ kindling is a common pre-clinical model of temporal frontal epilepsy in mice, which allows to investigate the effects of substances on the epileptogenesis of convulsions in the course of kindling development (administration of substances from the prophylactic scheme), as well as on the generation and generalization of convulsions in animals with fully developed kindling (administration of substances in the therapeutic scheme). The model of the PTZ kindling reproduces the GABA-receptor (GABA-R) dependent epileptogenesis [10].

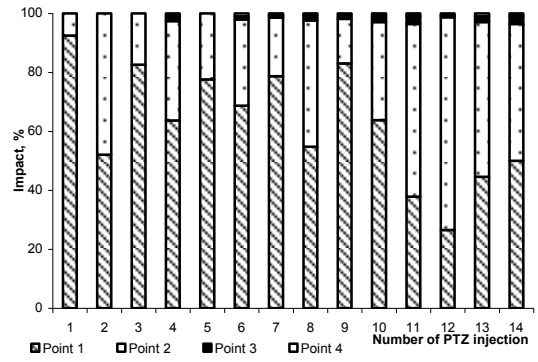
The kindling state is modeled by repeated PTZ administration to animals of a dose that does not cause convulsive effect (subdose). Such an effect increases the gradual convulsive "readiness" of the brain to the effect of convulsants. This is manifested in the fact that previously inactive subconvulsive doses of PTZ caused seizures, the severity of which increases over time and ends with generalized clonic-tonic seizure. The kindling model in the development of chronic epileptogenesis is also considered as a pharmacologically resistant form of the epileptic syndrome [11]. Possible mechanism of drug resistance include the negligible to lack of effect of antiepileptic drugs (narrow therapeutic index, low level of effective concentration of the drug in the brain, reduced response to the

drug in repeated or prolonged use as a result of adaptive body reaction). These indicators can be modeled in experiments on animals. This approach only to some extent reflects the natural situation that occurs in the clinic as the resistance of the therapy of epilepsy depends on the course of the disease itself, and the pharmacodynamic mechanisms (primary resistance) and the pharmacodynamics of drugs (secondary resistance), although the search for effective pharmacological agents in such conditions is definitely the basis for their successful application in medical practice.

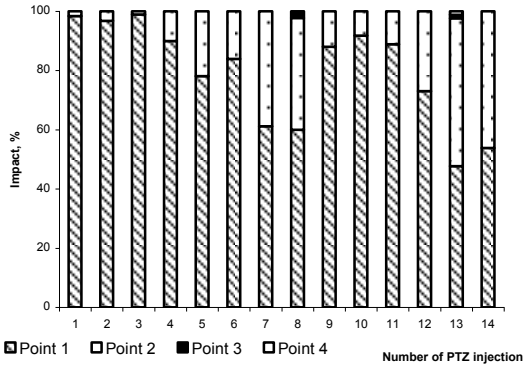
The analysis of initial experimental data showed that the obtained data can not be described by the normal (Gaussian) distribution law, since their predominant part has different indicators of asymmetry and excess (in 1,5-2,8 times the values of the corresponding standard deviations). In this regard, for the corresponding data of the control and experimental groups of animals, the values of the difference were estimated on the basis of the non-parametric Wilcoxon-Mann-Whitney criteria.

It should be noted that seizures of the fifth (highest) severity degree were not observed in any experimental animal group, and seizures of the fourth severity were observed only in single animals and only on the terminal days of the experiment. For seizures of the first to third severity degree the calculated values of the difference significant level do not show an adequate level (p>0,2-0,6), which does not allow us to conclude that the effects of the administered doses of propoxazepam on the development of convulsions during the chronic PTZ administration have not been demonstrated. For indicators of the number of second-degree severity seizures in the group of animals receiving propoxazepam at a dose of 2.0 mg/kg the calculated significance levels were less than 0.05 (eighth and ninth administration).

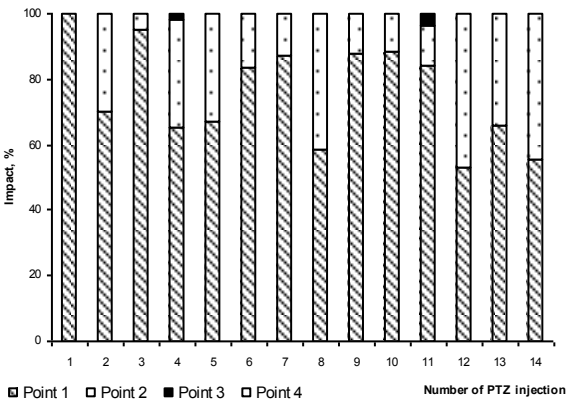
For animals of control (Fig. 1A) and experimental groups (Fig. 1B-D), there was a decrease in the latent time of seizures manifestation of the first severity degree. In the control group of animals and in the group receiving propoxazepam at a dose of 0.2 mg/kg, the minimum value of the latent time of the first degree of severity convulsion appeared on the 8$^{th}$-11$^{th}$ day of PTZ administration, what can be regarded as the absence of significant influence of the compound on development prolonged neurodegenerative changes.



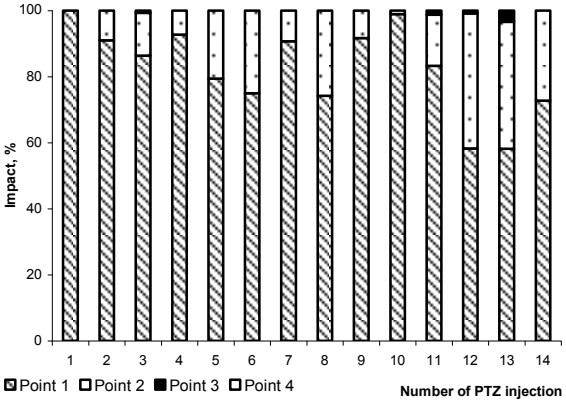

A



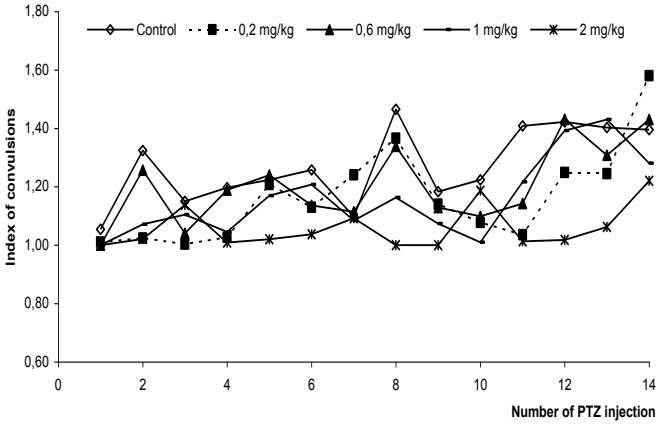

B




*C*




*D*




*E*

*Fig. 1. Change in the partial contribution of convulsions of various severity degrees to the total number of mice attacks convulsions in the control group of animals (A) and in experimental groups (B-E) treated with propoxazepam (0.2-0.6-1.0-2,0 mg/kg, respectively).*

In the control group of animals before the 8th PTZ injection, there is also the occurring of convulsions of the third and fourth degree of severity, however, these indicators begin to stabilize only after the 11th administration (Fig. 1, A). In the experimental group of animals (0.2 mg/kg of propoxazepam), seizures of the third and fourth degree of severity occur later (13th administration). In addition, convulsions of the third type of severity (Fig. 1D, E) are observed even when high doses of propoxazepam were administrated (1.0 and 2.0 mg/kg). Obviously, the effect of propoxazepam even in high doses on GABA-R completely does not compensate the functional changes in other mediator systems (NMDA-, aspartate and glutamate), which are involved in the processes of kindling development.

In terms of severity of kindling-epilepsy pathological process development the partial contribution of each of convulsions types to general seizure spectra is indicative. To relatively assess the changes in qualitative composition of seizures attack, the participation of each type of convulsion was determined in relation to the total amount of seizures (Fig. 1A-E). This method of data transformation not only reflects the individual contribution of each type of convulsion, but also gives an idea of the depth of influence of the long-term administration of PTZ on the balance of inhibitory and excitation systems.

In animals of the control group (Fig. 1A) during the development of the kindling-state a gradual decrease in the number of first-degree convulsions with a simultaneous increase in the representation of the higher-severity seizure arising from functional changes in the central nervous system is observed. The fourth administration leads not only to increase of third group convulsions contribution increase, but also the appearance (to 3-6%) of more severe components. After the sixth administration, their presence in the seizure spectrum becomes constant, although the total contribution does not exceed 6% (Fig. 1A).

In general, the assessment of the partial contribution is more indicative for the effect of the propoxazepam administered doses on the spectrum of seizure attack (Fig. 1B-E). In the spectrum of convulsive activity of animals that received propoxazepam in a dose of 2.0 mg/kg, practically there is no manifestation of attacks of even a second degree of severity, indicating a direct effect on GABA-R.

One of the classical parameters that characterizes the development of the kindling-state is the index of convulsions (*I*), which is both normalized and the average indicator of the contribution of each convulsion type to the general state of the kindling, which is registered.

Calculated values of the seizure index do not undergo significant changes and do not exceed the value of 2.0 (Fig. 2). Both in the control group and in the experimental groups of animals receiving low doses of propoxazepam (0.2 and 0.6 mg/kg), this indicator does not show statistically significant differences, and only in the group of animals receiving propoxazepam at dose of 2.0 mg/kg, its tendency to a significant increase is not observed.

In general, the analysis of primary data (latent time of convulsive attacks development, their number and degree of severity) does not allow to make conclusion about the amount of contribution of propoxazepam to inhibit the development of the kindling state in experimental animals. However, the variance analysis of these data from control and experimental groups characterizes the effect of the total factor of the propoxazepam administered doses (0.2-2.0 mg/kg) at 23 % and leaves about 77 % of other factors (Table 1). At first glance, this is a low value (despite a statistically significant result with $p = 0.002$) for a compound that has shown a high anticonvulsant effect in acute PTZ experiments, especially given the statistically significant differences in severity of convulsions. However, this may be due to the lack of exposure to low doses of propoxazepam, the excessive variability of control values, or a combination of mutually exclusive factors (for example, the lack of efficacy of propoxazepam in the terminal stages of the kindling state).

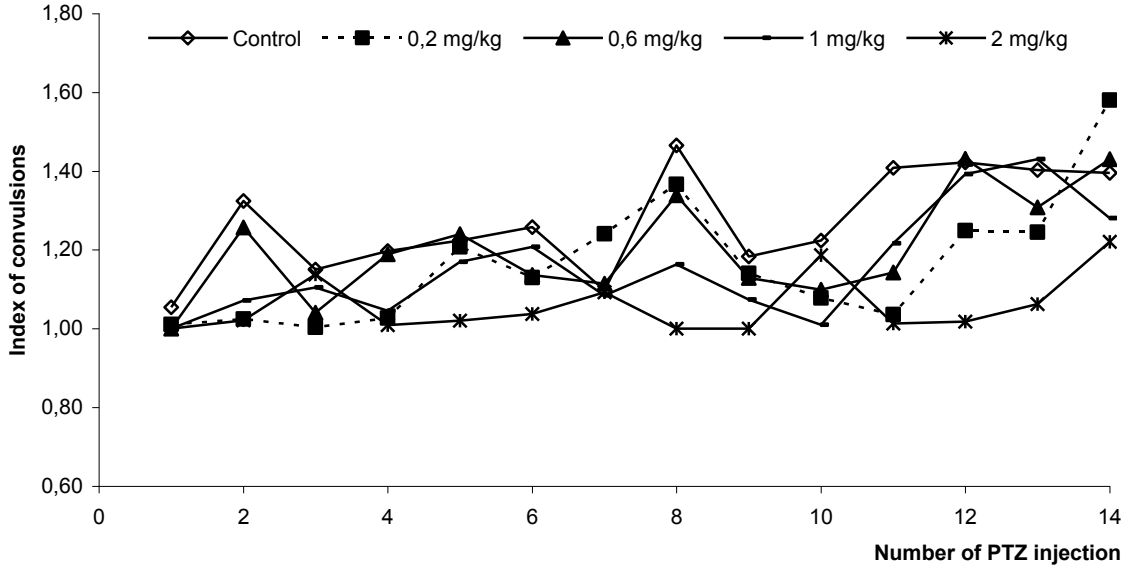



*Fig. 2. Change in the value of the index of convulsions (I) of the control and experimental (propoxazepam in doses of 0.2-2.0 mg/kg) animal groups during the kindling state development.*

Separation of a complex factor (a combination of propoxazepam doses) into separate subgroups and an assessment of the effect of each dose administered (relative to the control group) can reveal an increase in the dose effect of the propoxazepam (from 11.8% to 51.9% for doses 0.2 -2.0 mg/kg). On the basis of the calculated values of the contribution of these factors, it is possible not only statistically significantly (for each individual group, the probability level was <0.001) to detect the existence of dose-dependent intermittent effects of propoxazepam, but also to explain the impossibility of detecting this effect by other methods of data analysis.

Table 1

**Dispersion analysis of the effects of doses of propoxazepam on the process of kindling formation in mice.**

| Group | Factor dispersion (factor varience) | Non-factor dispersion (group varience) | The total dispersion | The contribution of factors taken into account,% | Contribution of unaccounted factors,% | The calculated level of statistucal significance, p |
|---|---|---|---|---|---|---|
| Estimation of the overall effect of propoxazepam | | | | | | |
| (control, doses 0.2 mg/kg, 0.6 mg/kg, 1.0 mg/kg, 2.0 mg/kg) | 1.114 | 0.336 | 1,450 | 23 | 77 | 0,002 |
| Estimation of the influence of dose-dependent factors on the propoxazepam administration during the development of kindling process | | | | | | |
| 0.2 mg/kg | 0.077 | 0.574 | 0.651 | 11.8 | 88.2 | <0.001 |
| 0.6 mg/kg | 0,032 | 0.463 | 0,495 | 6.5 | 93.5 | <0.001 |
| 1.0 mg/kg | 0.086 | 0.457 | 0,544 | 15.9 | 84.1 | <0.001 |
| 2.0 mg/kg | 0.318 | 0,295 | 0.613 | 51.9 | 48.1 | <0.001 |

It has been found that administration of low doses (0.2-0.6 mg/kg) of propoxazepam during the period of formation of the kindling does not have a significant anticonvulsant effect, although it inhibits the manifestation of the third and fourth severity degree convulsions. Propoxazepam high doses (1.0-2.0 mg/kg) inhibit the development of high severity degree seizures; at the administration of high doses (2.0 mg/kg), with practically no manifestation of attacks even a second degree of severity. According to the results of the dispersion analysis, the contribution to the factor of the propoxazepam dose on the model of kindling-epilepsy ranges from 11.8% to 51.9% for doses 0.2-2.0 mg/kg).

The presented data also indicate that there is no reduction in the response to propoxazepam at doses of 0.2-2.0 mg/kg under repeated and continuous administration schemes, which indicates that there is no experimental pharmacodynamic resistance to the antiepileptic action of the substance. According to the literature [12], valproate - one of the mostly used antiepileptic drug - in high doses (100-200 mg/kg) in chronic oral prophylactic administration maximally suppresses the development of generalized clonical-tonic convulsions of a 4$^{th}$ severity degree and only in a small number of mice prevents the development of local clonic convulsions with severity of 1-2$^{th}$ degree. The deficiency of valproate can also be attributed to the weakening of its anticonvulsant effect by 8-11% to 21 days of kindling formation, which may indicate the development of tolerance to valproate in high doses with prolonged use.

PTZ-induced kindling may be related to permanent attenuation of inhibitory function of GABAergic system in the brain. [10] Repetitive single dose application ends up with decreased GABAergic activity [13]. According to this suggestion the functioning of the $GABA_A$-receptor in the CNS can be examined *in vivo* by estimation of the competitive effects of benzodiazepines and seizure-inducing agents (pentylenetetrazol, strychnine, and picrotoxin). On the basis of dose–effect curves, using comparative quantile analysis for chemoconvulsants with different mechanisms of action, we showed different stages of propoxazepam interaction with GABA and glycine receptors under *in vivo* conditions [4].

**CONCLUSIONS**

It was found that propoxazepam did not lead to reliable changes of seizures manifestations, but limited second type seizures development, reducing their quantity. The reliable redistribution in seizures quantity with two and four points with simultaneous increasing of higher severity degree seizures was noted. At the same time seizures of fourth severity points were diminished. For animals of control and experimental groups the reducing of first and second seizures type latent time. With propoxazepam administration reducing of higher severity seizures (3 and 4 points) is in accordance with reducing of the latency time for seizures of $1^{th}$ and $2^{nd}$ types. In the late period of kindling development the increase of seizure index in the experimental group of animals was registered, what can be due to the glutaminergic system activation in the terminal stage of the epileptic activity formation.

**References**

1. Marc A. Dichter. Basic mechanisms of epilepsy: targets for therapeutic intervention. Epilepsia. 1997; 38(Suppl. 9): 2-6.

2. John G.R. Jefferys. Advances in understanding basic mechanisms of epilepsy and seizures. Seizure. 19 (2010) 638–646.

3. Andronati S., Semenishyna E., Pavlovsky V., Simonov Y., Makan S., Boyko I., Burenkova N., Gdaniec M., Pascal Cardinael, Jean-Philippe Bouillon, Mazepa A. Synthesis, structure and affinity of novel 3-alkoxy-1,2-dihydro-3H-1,4-benzodiazepin-2-ones for CNS central and peripheral benzodiazepine receptors. European Journal of Medicinal Chemistry 2010; 45: 1346–1351.

4. Sato M. Kindling: An Experimental Model of Epilepsy. Psychiatry and Clinical Neurosci., 2008; 36: 440-441.

5. Urbach V. Yu. Statistical analysis in biological and medical research. M. Medicine, 1975. 297 pp.

6. Reder A., Andronati S., Golovenko N., Pavlovsky V., Kabanova T., Khalimova E., Larionov V., Voloshchuk N. UA Patent, UA № 115205. 2017.

7. Golovenko N., Larionov V., Reder A., Valivodz' I. An effector analysis of the interaction of propoxazepam with antagonists of GABA and glycine receptors. Neurochemical Journal. 2017; 11 (4): 302–308.

8. Golovenko N., Reder A., Larionov V., Valivodz` I. The effect of propoxazepam on development 0f thiosemicarbazide-induced GABA-deficientseizures in mice. Clinical pharmacy, 2017; 21: 34-40.

9. Özlem Ergül Erkeç, Okan Arihan. Pentylenetetrazole kindling epilepsy

10. Corda M.G., Orlandi M., Carboni G., Frau V., Giorgi O. Pentylenetetrazole induced kindling in the rat: Effects of GABA function inhibitors. Pharmacol Biochem Behav., 1991; 40: 329-333.

11. Sharma A.K., Reams R.Y., Jordan W.H., Miller M.A., Thacker H.L., Snyder P.W. Mesial temporal lobe epilepsy: pathogenesis, induced rodent models and lesions. Toxicol Pathol., 2007;35: 984–999..

12. Löscher W. Critical review of current animal models of seizures and epilepsy used in the discovery and development of new antiepileptic drugs. Seizure. 2011; 20: 359-368.

13. Corda M.G., Orlandi M., Lecca D., Giorgi O. Decrease in GABAergic function induced by pentylenetetrazol kindling in rats: antagonism by MK-801. J Pharmacol Exp Ther 1992;262: 792-800.