Influence of Particle Size on The Anticonvulsant Activity of Propoxazepam.

Anatoliy Reder. Vitalii Larionov, Nikolay Golovenko, Sergey Andronati

A.V.Bogatskiy Physico-Chemical Institute of NAS of Ukraine, Odessa

vitaliy.larionov@gmail.com, n.golovenko@gmail.com

Abstract

The granulometric composition of new analgesic substance (7-bromo-5-(o-chlorophenyl)-3-propoxy-1,2-dihydro-3H-1,4-benzodiazepin-2-one) was characterized by methods of crystalls microscopy and lazer diffraction,. The single polymorphic phase demonstrated by differential scanning calorimetry, X-ray diffraction analysis, Raman and IR-spectroscopy. On the base of the anticonvulsive effect on mice in compare to coarse sryctalline sample the the conclusion about the higher pharmacological effect for the disperse sample had been demonstrated.

Keywords: Propoxazepam, Particle Sizes, Molecular Structure, Anticonvulsant Activity

Introduction

One of the main tasks of pharmacy and clinical pharmacology is the creation of optimal conditions for the absorption of the drug taken orally. On this depends the speed of the onset of the effect, and the establishment of maximum concentration (in time and absolute value) and other factors [1]. To improve the properties of active pharmaceutical ingredient (API), various technologies are used: a) reducing the particle size to increase the degree of solubility of the drug substance and the absorption area; b) stimulation of dissolution in the parietal fluid of the gastrointestinal tract by means of special carriers; c) formation of an aqueous-soluble complex with a drug agent; d) use of a prodrug or active metabolites with a high dissolution index; e) the effect on the crystal lattice of the drug substance. [2]. Each of the best methods has its advantages and limitations and is selective for improving the drug dosage forms. Given the structure and physico-chemical properties of the compound, the most appropriate task is to reduce the size of its particles, since the dissolution rate of the preparation is directly proportional to the surface area and inversely proportional to the particle size of the substance.

Consequently, the particle size (granulometric composition) of the API is an extremely important physical characteristic of chemical compounds used to create medicines with a specified target quality profile. Valuable also are knowledge of morphological features in connection with information about the chemical composition and form of particles. Particles change of the substance can lead to changes on pharmaceutical and, as a concequence, to pharmacological indicators as well as therapeutic efficiency of the medicines. Because of this the determination and control of such properties have essential importance in the process of innovative drugs and their generic analogs.

Propoxazepam, 7-bromo-5-(*o*-chlorophenyl)-3-propoxy-1,2-dihydro-3H-1,4-benzodiazepin-2-one, in the models of nociceptive and neuropathic pain showed significant analgesic activity [3]. Similar to gabapentin and pregabalin, which are well-known anti-epileptic drugs used in general medical practice in the treatment of neuropathic pain, propoxazepam also has an anticonvulsant effect [4], which explains the analgesic component of the pharmacological spectrum.

In the present study, we prepared propoxazepam of various particle sizes and assessed the effects of particle size the on physiological activities, such as the anticonvulsant activity in mice.

Materials and Methods

Chemicals

In the study the sample of propoxazepam crystalline form, obtained according to the previously described method of synthesis and purification [5], was used. 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%).

Preparation of particles of propoxazepam

Recrystallization is also an operation in the manufacture of pharmaceutical compounds. It is often a key isolation step or a purification process in the synthesis of an active pharmaceutical ingredient [6].

Propoxazepam crystalline form (form A, PFA) was obtained by recrystallization of propoxazepam from 96% ethyl alcohol. A portion of the technical PCF was dissolved in boiling ethyl alcohol (from the ratio of 1g of the substance per 8,0 ml of alcohol), the hot solution was filtered and allowed to crystallize with stirring and slowly cooling for 12 hours. The filtered product was washed with ethyl alcohol and dried in a drying cabinet at 100-105 °C for 24 hours. The yield of the PFA was 80%.

The dispersed form of propoxazepam (form B, PFB) was obtained by dispersing the PFA sample in the following way [7]: to boiling ethyl alcohol with stirring, PFA was added in a ratio of 1:8 by weight. The resulting suspension was kept with stirring until the substance was completely dissolved and the hot solution was poured into a 1,5-fold (in relation to the volume of ethanol) volume of water. The precipitate was immediately filtered through a Nutch filter (pore size 16) and washed on a filter with cold water (2x15 ml). The resulting PFB compound was dried in a drying cabinet at 110-120 °C for 2 days. The yield is 95%.

Measurement of particle size

Microscopy of crystals. The method of microscopy with software is accessible and accurate enough to determine the linear dimensions and shapes of particles, and therefore it is recommended for assessing the quality of pharmaceutical substances [8].

Laser diffraction. A water-alcohol (10:1 v/v) sample dispersion was introduced into a dispersant (with reduced volume), covered with a lid and at a stirring speed of 2000-2500 rpm, and taken the readings (Mastersizer 3000 «Malvent», lazer quenching 8-13 %, solution of sodium dodecil sulphate - 1%).

Differential scanning calorimetry.

Sample preparation: the sample was placed in an aluminum analysis bowl, covered with a lid under a press and placed in a sample cell (DSC Q2000 "Thermo Sientific"). Heating was carried out in the range of 40-340 °C, with a heating rate of 5 °C/min.

Molecular structure of PFA and PFB.

The molecular structure of of propoxazepam was measured by an Infrared (IR) and spectroscopy of raman scattering (RS) of crystals and mass spectrometry (DXR Raman Microscope "Thermo Sientific"; lazer power 24 mW, wavelenght 780 nm, measuring range in antistocks area 3370-100 sm^{-1}). Monocrystalline X-ray studies were carried out on a diffractometer «Xcalibur3» (Molybdenum radiation, graphite monochromator, CCD «Sapphire3» detector, ω-scanning, 2**Error! Reference source not found.**макс= 50°)

Measurement of anticonvulsant activity in mice.

Anticonvulsant properties propoxazepam (a predictor of antiepileptic action and antinociception in neuropathic pain) was studied in models of pentylenetetrazole and picrotoxin convulsions in doses causing effects in 95% of white mice (120 mg/kg and 6,5 mg/kg, respectively). The protective effect of PFA and PFB was determined by the magnitude of the effect (the number of surviving animals), which was recorded for 2 hours after subcutaneous administration of a convulsive agent. During the observation period, the time of onset of myoclonic tremor, generalized seizures in the form of tonic extension, the number of these types by the court, and the time of death of the animal were determined. The quantitative criterion for the protective effect of the compound was the ED_{50}, in which the probability of manifestation of a protective action in 50% of the animals is the greatest.

Statistical analysis

The obtained values are shown as the means ± standard error (SE). A one-way analysis of variance (ANOVA) with post hoc test was performed to determine the differences between the groups. The level of significance was 0.05.

Results and Discussion

Characterization of crystals by microscopy. Propoxazepam is a white powder. Crystals are tetragonal prisms containing fine crystalline agglomerates. The crystal sizes are 20-120 microns for PFA and less than 20 microns for FDS. (Fig. 1, magnification × 500). Optical-microscopic observations of single crystals of the compound showed that the transformation of PFA → PFB proceeds topochemically, that is, through the nucleation of the product phase and the progress at the crystal interface, as described for other substances [9]. The phase transition in propoxazepam can be described within the framework of the cooperative mechanism of polymorphic transformations [10]. For the cooperative mechanism, in contrast to the atomic mechanism, the absence of a definite shape of the germs is characteristic, as well as the rapid advancement of the interface along the crystal with a non-constant rate.

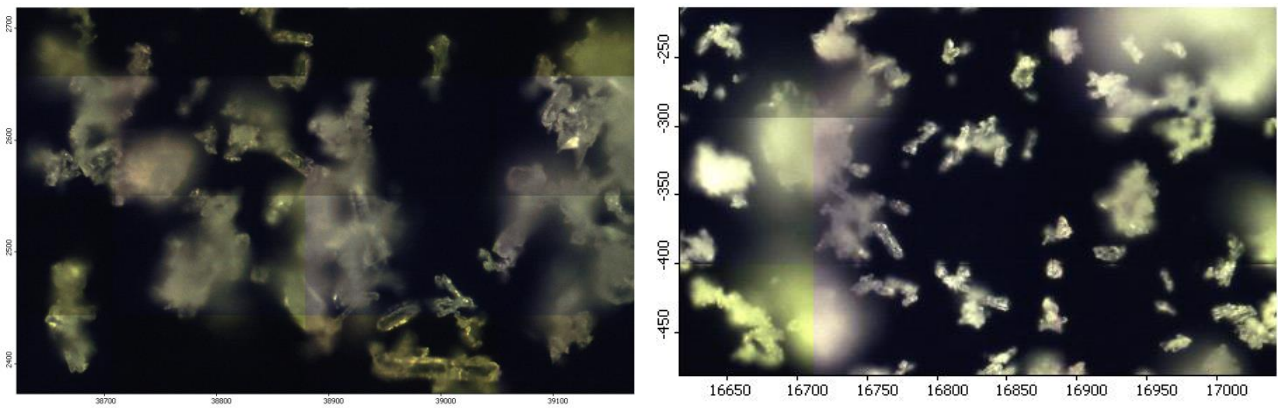


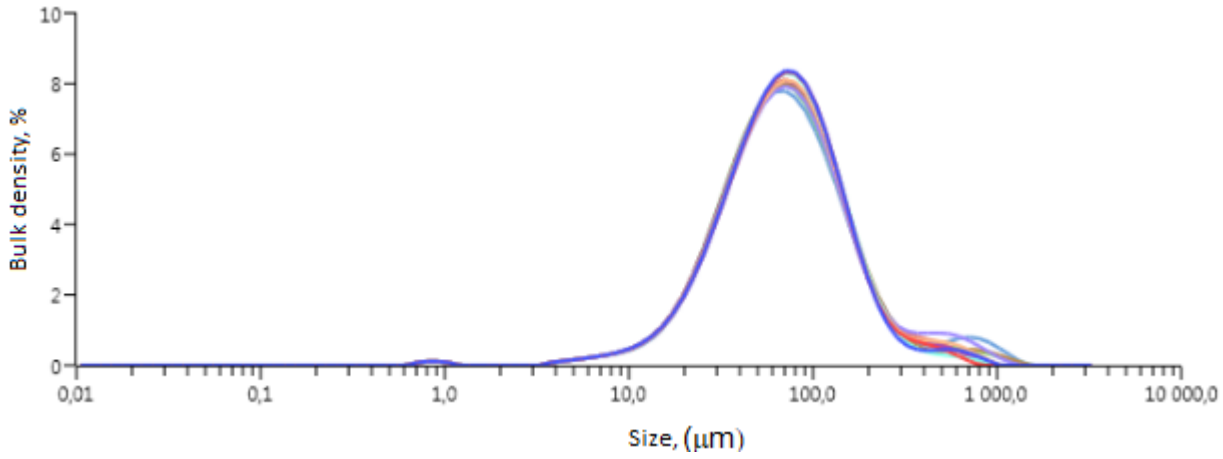
Fig. 1. Microscopy of crystalls of samples PFA and PFB (magnification × 500).

Laser diffraction granulometric composition. From the results of the conducted studies it can be concluded that there are differences in the morphology of PFA and PFB. In these samples, crystal size differences are observed: the PFA compound is 20-120 microns, and the FDS is less than 20 microns, but the crystal structure is similar (Table 1).

Table 1. The percentage composition of particles of different size in samples PFA and PFB.

Series	PFA			PFB		
The bulk fraction of particles with a size less than D_x(%)	$D_{x(10)}$	$D_{x(50)}$	$D_{x(90)}$	$D_{x(10)}$	$D_{x(50)}$	$D_{x(90)}$
The average particle size (microns)	25,2	69,4	206	9,9	29,4	122
Standard deviation	0,62	0,66	1,56	0,65	1,03	8,50
Relative standard deviation (%)	6,40	1,99	7,65	10,01	7,98	2,18

The investigated samples differ significantly in the granulometric composition: in PFA, 50% of the volume fraction of particles have a size less than 69,4 microns, whereas in PFB this figure is almost half that of 29,4 microns. The PFB pattern behind the representation of particles of different sizes is more homogeneous - almost 80% of the particles have a size within 112 microns, whereas for the PFA this range is much wider and is almost 180 microns. Since the particle size determines the area of their surface and, accordingly, the dissolution rate, this granulometric composition will have more optimal biopharmaceutical properties (degree, speed and uniformity of dissolution). It should also be noted that the PFB sample, in contrast to the PFA sample, has a smaller spread in the sizes of relatively large particles (the relative standard deviation for the volume fraction of particles smaller than 122 microns is 2,18, while in the PFA sample this value is more variable - 7,65), which can also have a positive effect on the ability of the compound to be absorbed in the gastrointestinal tract. The distribution of the particles of the compound and by their dimensions is shown in Fig. 2. The data obtained confirmed the results of microscopic studies of both forms of API. Indeed, the form of compound PFA is represented to a greater extent by particles of 25-206 microns in size (Fig. 2, a), while PFB is 10-122 microns (Fig. 2, b).



A

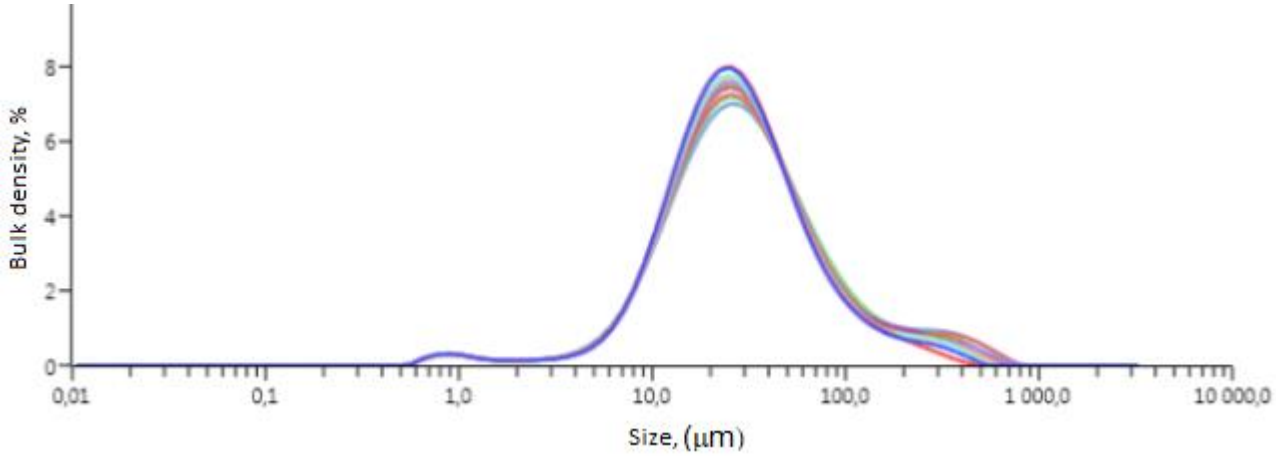


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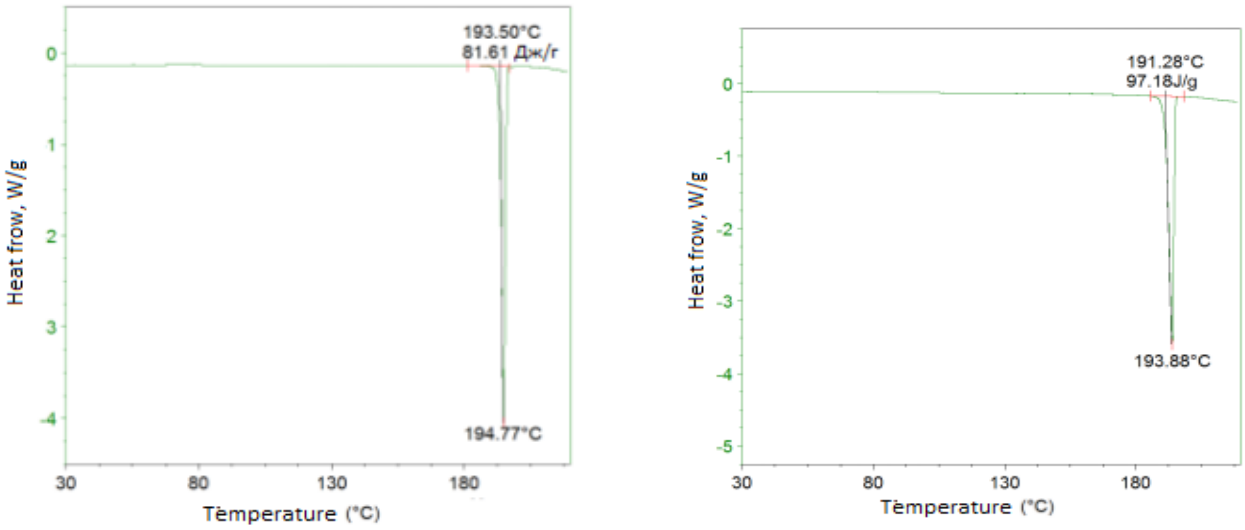
Fig. 2. The distribution of the particles for samples PFA (A) and PFB (B) determined by lazer diffraction.

Differential scanning calorimetry studies. The PFA derivatogram contains one narrow endothermic peak at 193.5-194.8 °C, corresponding to the melting of the sample. On the derivatogram of PFB, a peak in the range of 191.3-193.9 °C is observed. (Fig. 3). The decrease in the melting temperature of the sample is due to differences in crystal sizes of the two samples.



PFA PFB

Fig. 3. Differential scanning calorimetry studies of samples PFA and PFB.

X-ray phase studies. Both samples differ significantly in the content of the corresponding polymorphs. So the triclinic polymorph - in the composition of CCS ~ 4 times more, (3,5 and 13 mass.%, respectively). The identification of difference peaks allowed us to assume that the samples contain another triclinic polymorph with the parameters of the cell a = 10.8967Å, b = 15.1677Å, c = 11.8963Å, ▪ = 106.212 °, ▪ = 96.739°, ▪ = 88.481 °, V = 1874.9 Å3, that is, the samples are three-phase. For the monoclinic modification, the Rietveld method was used to calculate the anisotropic microstructural characteristics, which gave similar results for both samples characterizing the particle size and shape. The results in Table 2.

Table 2. microstructural characteristics of samples PFA and PFB determined in X-ray phase studies

Sample	Phase	Content, mass%	Average particle size/ microcurrent
FDS	Triclinic polymorph	3.5(10)	28 / –
	Monoclinic polymorph	96(3)	155(52_{010}–264_{-101})/0.27
CCS	Triclinic polymorph	13.4(7)	28 / –
	Monoclinic polymorph	87(2)	119(67_{010}–223_{-101})/0.37

It was found that for the monoclinic polymorph in both samples, the crystals are plate-like in the coordinate plane (010). The structure of the triclinic polymorph is layered, that is, that is, they alternate layers of triclinic and monoclinic modification due to the fusion of lamellar crystals, with the plane of adhesion (micro-twinning) being the (010) plane of the monoclinic polymorph. The twinning of compound **I** in the crystal was proved by X-ray diffraction analysis [4].

Molecular structure of PFA and PFB.

Raman scattering (RS) spectroscopy of crystals. The vibrational spectra of two samples have the same set of bands by position and intensity. The correlation between the RS (combination scattering, CS) is 97,0%, the IR spectra are 98,8%, that is, with a high probability, these spectra can be considered identical. RS spectra of two samples also have the same set of bands by position and intensity. The correlation between spectra in the 3400-400 region is 98,4%, in the 1800-400 cm^{-1} - 98,5% (Fig. 4).

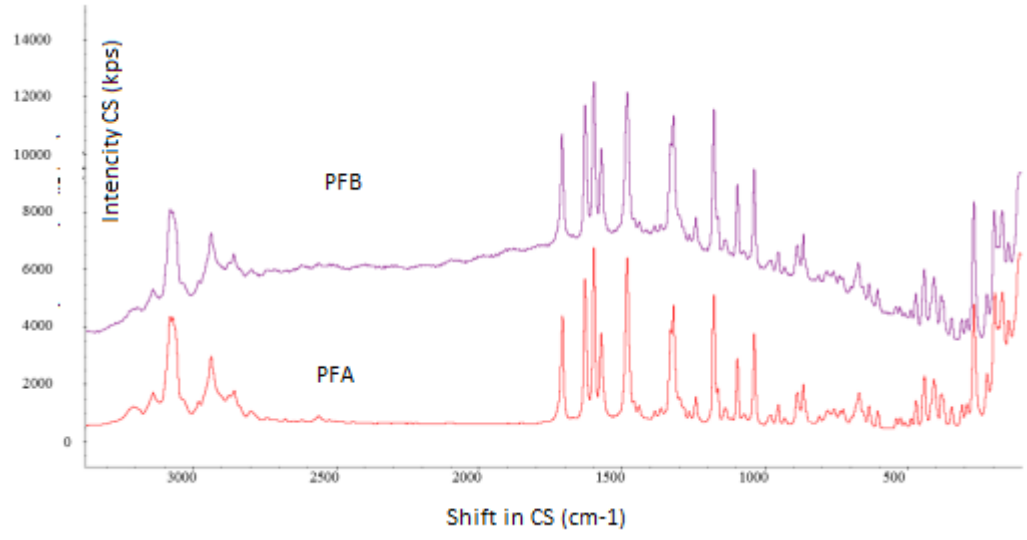
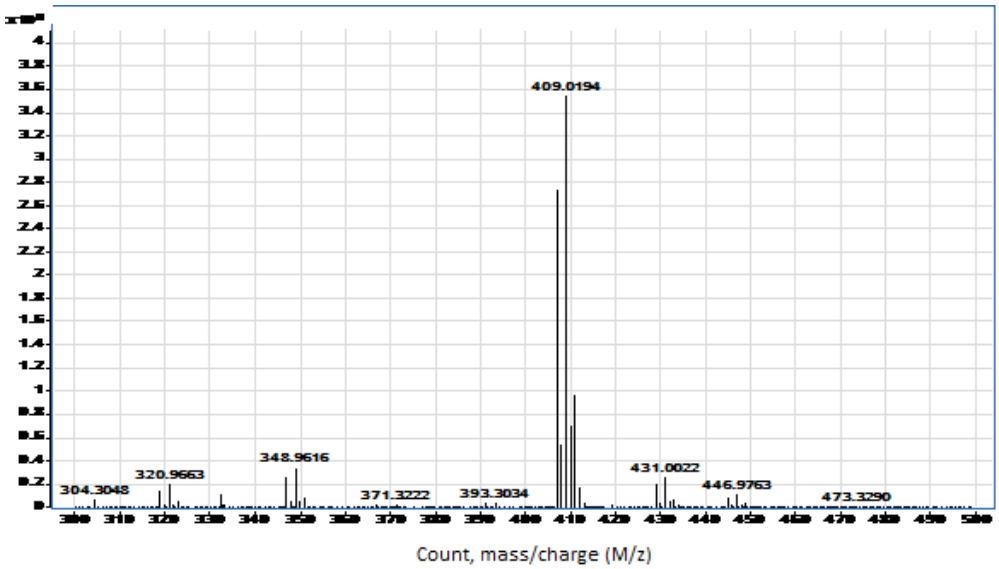


Fig. 4. Combination scattering spectra for samples PFA and PFB.

Mass spectrometry was carried out on a chromatography-mass spectrometer in a combined HPLC-MS system, a liquid chromatograph 1260 Infinity with a detector of 6530 Accurate Mass Q-TOF (Agilent Technologies, USA) under the following conditions: a column of stainless steel (10 cm x 4,6 mm, filled with silica gel

octadecyl silyl for chromatography with a particle size of 3,5 microns, the mobile phase of acetonitrile-formic acid (0,% aqueous solution) -methanol (35:15:50), elution rate 0,5 cm^3/min, column temperature 40 °C , sample volume 1 mcl, analysis time 10-20 min, ion current detection, spot ionization - double electrospray at atmospheric pressure, carrier gas temperature 300 °C, fragmentation energy 270 W. In the mass spectra of two samples, there is an identical set of signals of a molecular ion ($M^+ H^+$) 409 m/z and some fragment ions (PFA Fig. 5, A and PFB Fig. 5, B).



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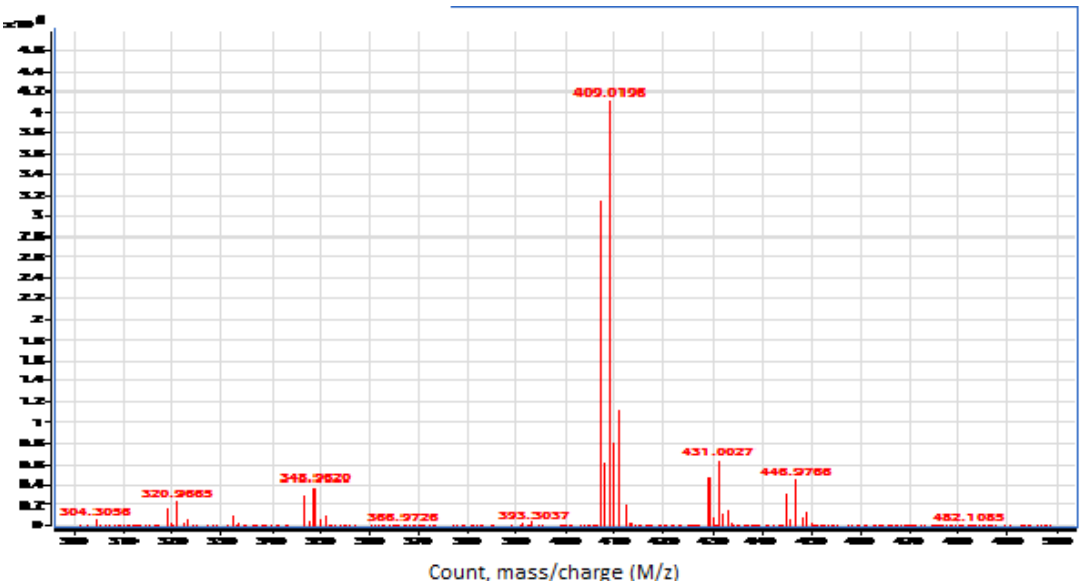

B

Fig. 5. Mass spectrometry for samples PFA (A) and PFB (B)

Evaluation of the anticonvulsant activity of propoxazepam particles in the mice.

According to ED_{50} the compounds PFA and PFS significantly differ (Table 3). According to the test of pentylenetetrazole and picrotoxic seizures, the PFB activity exceeded the similar indicator of PFB in 5,5 and 1.5 times, respectively. However, the slope of the "dose-effect" curve for both forms is close (0,821 and 0,717 for pentylenetetrazole and 0,783 and 0,639 for picrotoxin), indicating a similar mechanism for the realization of their protective effect on the antagonism with these convulsive agents.

Table 3. Parameters of anticonvulsive action of propoxazepam two samples (PFA and PFB) in mice.

Convulsant agent	Indicator	PFA	PFB
Pentylenetetrazole (120 mg/kg)	ED_{50},mgrkgr (M±m)	0,914±0,376	0,169±0,042**
	The slope of the "dose-effect" curve	0,821	0,717
	The parameters of inhibitory action (in an equivalent dose of 0,1 mg/kg)		
	Latent period of myoclonic seizures, min.	2 (1÷2)	2,5 (2÷6,75)
	Number of seizures	3 (3÷6,25)	2 (1÷2)
	Latent period of development of tonic convulsions, min.	2 (2÷2)	3 (2÷4)
	Number of tonic seizures	23,5 (16,5÷29,25)	17 (5÷44)*
	Life hour after administration of convulsant agent, min.	8 (7÷14,25)	15,5 (12÷20)
Picrotoxin (6,5 mg/kg)	ED_{50}, mg/kg (M±m)	1,67±0,09	0,366±0,068**
	The slope of the "dose-effect" curve	0,789	0,639
	The parameters of inhibitory action (in an equivalent dose of 0,5 mg/kg)		
	Latent period of myoclonic seizures, min.	6 (5÷6)	8,5 (8÷9)
	Number of seizures	55 (30÷56)	10 (8÷13)**
	Latent period of development of tonic convulsions, min.	10,5 (8,25÷12)	13 (11÷14)
	Number of tonic seizures	40 (35÷44)	15 (8÷25)
	Life hour after administration of convulsant agent, min.	25 (24÷32)	37 (37÷37)*

Results are expressed as a mean ± SEM. n = 6-8.

* - p < 0.05; **p < 0.01: values are significantly different from PFA

Both propoxazepam forms (PFA and PFB) for ED_{50} significantly differ (Table 3). According to the test of pentylenetetrazole and picrotoxic seizures, the PFB activity exceeded the similar indicator of PFA in 5,5 and 1.5 times, respectively. However, the slope of the "dose-effect" curve for both forms is close (0,821 and 0,717 for pentylenetetrazole and 0,783 and 0,639 for picrotoxin), indicating a similar mechanism for the realization of their protective effect on the antagonism with these convulsive agents.

Both forms of propoxazepam (in an equivalent dose of 0,1 mg/kg) increase the latent time of onset of development of individual components of the seizure attack, raising the threshold of epileptogenic ability of the brain. The displacement of the median time of development of clonic and tonic manifestations of convulsive seizure is not statistically significant and is within the interquartile range, however it is observed for all components of seizures when both chemoconvulsants are used. An increase in 0,25-0,5 times the latent time of seizure development is observed when animals are injected with PFB.

A significant difference in the effect of PFA occurs when comparing the number of myoclonic and tonic convulsions. Their number reflects the balance of the processes of excitation and inhibition in the brain at the stages of the onset of a convulsive attack and can characterize the propensity of the central nervous system to restore balance due to the action of the inhibitory system in the conditions of development of paroxysmal activity. Practically for all parameters of inhibitory action (in an equivalent dose of 0,1 pentylenetetrazole and 0,5 mg/kg), the PFB compound has advantages over PFA (Table 3).

These evidence support the data [4] that the inhibition of the development of pathological excitation by propoxazepam occurs primarily through GABAergic mechanisms. The use of selective chemoconvulsants also suggests the involvement of mixed GABA/glycine synapses, whose contribution to the overall effect, however, does not exceed 70%. The redistribution of the ratio of the myoclonic and tonic components of seizures that were induced by picrotoxin and pentylenetetrazole with an increase in the administered dose of propoxazepam also indicates the predominant participation of the GABAergic system in the mechanisms of its action. In the structure of seizures caused by strychnine, the leading component is the tonic component (over 50%), which indicates negligible involvement of glycine receptors in the pharmacological action of propoxazepam.

The increase of pharmacological action magnitude for PFB in compare to PFA can be explained by the dissolution increase what had been demonstrated by us earlier [7]. The results of the study of the dissolution of the maximum single dose in the amount of 3,0 mg PFB in 250 ml (which is similar to 12,0 mg of substance in 1000 ml) showed that the highest solubility of the compound is noted for a solution of hydrochloric acid with a pH of 1,2 (0,264 ± 0,044 mg/L). With increasing pH - in the acetic buffer solution (pH 4,5) the solubility of the compound decreases and amounts to 0,204 ± 0,018 mg/L, and at pH 6,8 phosphate buffer - 0,097 ± 0,009 mg/L. The decrease in the solubility of the PFB compound by almost 2,7 times with an increase in pH can be due to the fact that in the acid medium protonation of the compounds occurs with a partial increase in its polarity and, accordingly, the ability to dissolve in an aqueous medium. In general, the solubility of the PFB compound does not exceed 0,264 ± 0,044 mg/L (with a dissolution of 3,0 mg of substance in 250 ml, which corresponds to a solubility of 1 g of substance in 3,800 L), on the basis of which it can be attributed to compounds with low solubility. The obtained data on the solubility and bioavailability of PFB allows to reffer propoxazepam as the 2nd class of the biopharmaceutical classification system.

Conclusion

The disperse sample of the new biologicaly active compoud with anticonvulsive action was obtained using the method of solvent change. The obtained sample has a lower particle size but remains unnchanged in polimorphous form and chemical structure. The decreased particle size leads to increase in solubility and

pharmacological effect manifestation, that was demonstrated by atnticonvulsive effect determination in mice using picrotoxine and pentylenetetrazole as convulsive agents.

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