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Original Research Article

Dependence of anticonvulsant activity of 1-aryl-1, 5-dihydro-4H-pyrazole (3,4-d) pyrimidine-4-one derivatives on biopharmaceutical factors

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ABSTRACT

Background: We have synthesized three 5-R-1-aryl-1,5-dihydro-4H-pyrazole(3,4-d)pyrimidine-4-one derivatives that previously have demonstrated powerful anticonvulsant activity. A great number of physicochemical factors are known to influence on bioavailability and stability of active pharmaceutical ingredients. Therefore the purpose of research was to determine the effect of purification technology and dispersibility of 5-R-1-aryl-1, 5-dihydro-4H-pyrazole (3,4-d) pyrimidine-4-one derivatives on their anticonvulsant activity.
Methods: The anticonvulsant effect of this compounds was studied in a model of pentylenetetrazole-induced seizure in mice.
Results: The results obtained revealed the optimal solvent for recrystallization of compounds to be isopropanol: compounds, purified by recrystallization from isopropanol, had higher solubility in water and tween; also, they had a tendency to increase anticonvulsant activity. It was found that there is a significant dependence of the latter on compound's dispersion - the smaller the size of crystals the higher anticonvulsant activity.
Conclusions: The dependence of anticonvulsant activity of compounds on the degree of dispersion was proved: the smaller particle size the higher anticonvulsant activity. This can be explained by fast dissolution of fine-dispersed substances, thus increasing the bioavailability if the compounds studied.

Keywords: Anticonvulsants, Bioavailability, Pyrazole(3,4-d)pyrimidine-4-one derivatives

INTRODUCTION

Biopharmaceutical aspects are very important in pharmaceutical development. A great number of physicochemical factors are known to influence on bioavailability and stability of active pharmaceutical ingredients (API).

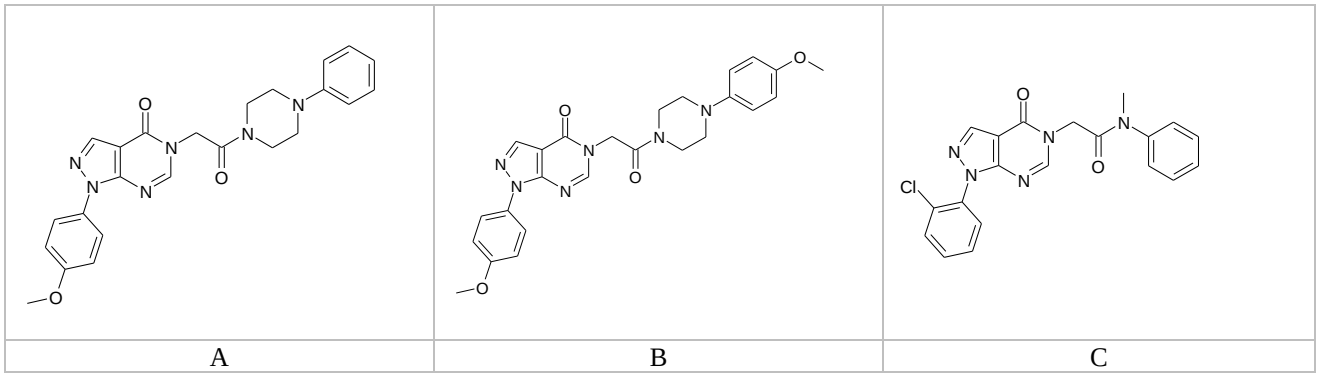
These include solubility, wettability, molecular structure, crystal shape and size, which considerably depend on the synthesis and purification technologies, in particular crystallization of organic compounds.[1-3]

Previously we have synthesized 5-R-1-aryl-1,5-dihydro-4H-pyrazole[3,4-d] pyrimidine-4-one derivatives, which according to the preliminary computer prediction could be promising anticonvulsants.[4,5] A pharmacological screening of anticonvulsant effect confirmed the prediction data and indentified compounds-leaders 1-3 among the synthesized pyrazolopyrimidine derivatives (Figure 1) that powerfully protected experimental animals against corazole seizures, being comparable with valproate sodium.[6,7]

Since the processes of synthesis of drug compounds, methods of their purification, drying and grinding have a

considerable effect on pharmacological action, an optimization of purification technology subject to current needs of commercial-scale production of pharmaceutical substances is important for the above compounds 1-3 in view of their promising features as potential APIs.

Purpose of the study was to determine the effect of purification technology and dispersibility of 5-R-1-aryl-1,5-dihydro-4H-pyrazole[3,4-d]pyrimidine-4-one derivatives on their anticonvulsant activity.



A	B	C

Figure 1: 5-R-1-aryl-1, 5-dihydro-4H-pyrazole[3,4-d]pyrimidine-4-one derivatives that demonstrate an anticonvulsant effect.

METHODS

Synthesis of compounds was performed according to the methodology described previously.[4] The synthesized compounds were crystallized from isopropanol, ethanol and mixture of ethanol and water (2:1). After recrystallization from different solvents melting points of compounds correspond to the one specified previously and do not result in the depression of melting point.[1] HNMR spectra are identical to those obtained previously.[4] The content of main compound is at least 99.5% (mass spectrometry).

Microscopic analysis was performed using "Konus-Academy" laboratory microscope with ScopeTek DCM510 camera. ScopePhoto™ application was used for viewing images and to measure linear dimensions in real time and on static image.

Microscopic studies were performed based on available methods to determine powder dispersibility. According to the established classification, all methods can be divided into the following groups:

- Mechanical particle separation including sieve and filtration analyses;
- Sedimentation analysis including fractional precipitation, desilting, sediment accumulation, weight sample collection;
- Dynamic methods based on separation in stream in vertical vessels and centrifuges;
- Single particle study including microscopic and ultramicroscopic analyses;
- Determination of specific surface including absorption method, by dissolution rate and others.

The sieve and microscopic methods are most common for express analysis of disperse composition of powders in measurable size range >0.5μm.

Each of these methods has advantages and disadvantages, which justify their reasonable practical use. Unfortunately, the sieve method does not provide reliable data on particle sizes due to agglomeration unavoidable at dry sieving. The microscopic analysis enables to determine parameters of highly dispersed powder more accurately.

Particle sizes were measured at observing specific visual fields. Specific visual fields were selected on the study powder sample by moving it to a distance exceeding a rectangle diagonal or a circle diameter limiting a visual field. An area on which the measurement took place and the number of particles are equal to the sum of their areas at observing certain visual fields. Identification of particles on certain visual fields was conducted by measuring a maximal chord in horizontal or vertical directions.

A particle was considered to pertain to the field observed if it was on one of halves of the field bounds.[8] For example, for a rectangle the particles within it, on its left vertical and upper horizontal sides, on the intersection of these sides and at another end of one of them were accounted. Particles on other sides and at the corners were not accounted.

The necessary particle size was obtained by sieving substances with sieves No. 125 (particle size 0.1μm).

Anticonvulsant effect of compounds 1-3 was studied in a model of pentylenetetrazole-induced seizure in mice.[9] The tests were conducted in 108 mature non-strain male albino mice weighing 20-25g. Animals were kept in the standard conditions of Vivarium of the National Pharmaceutical University's Central Scientific and Research Laboratory (NPU CSRL) in compliance with the sanitary and hygienic norms and principles of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. The experiments were conducted according to General Ethical Principles and Guidelines for Experiments on Animals.

The seizure syndrome was modelled in animals by pentylenetetrazole (corazole) administration ("Sigma", USA) (90mg/kg, s/c) 30 min after administering the study compounds.[10] Mice were randomly divided in 10 groups. Control animals (control, n=6) were given pentylenetetrazole (90mg/kg). Mice of comparator group (n=6) were given a reference anticonvulsant product valproate sodium ("Depakine", lot 472, Sanofi-Aventis, France) (300mg/kg) 30 min before administering a convulsant.

Anticonvulsant action was determined by administration of the study compounds. Compounds 1-3 (100mg/kg) in grinded (experiment a) and native (experiment b) form were administered to experimental animals into the stomach as suspended in Tween-80 30 min before administering pentylenetetrazole.

The anticonvulsant effect was assessed by latency period of seizures, number of animals with clonic and tonic seizures, and lethality. Animals were observed during 1 hour; if no seizures developed, the latent period was considered to be 60 min. Severity of seizures were evaluated according to a scale as follows: 1 - shudder, 2 - running in circles, 3 - clonic seizures, 4 - clonic-tonic seizures in lateral position, 5 - tonic extension, 6 - tonic extension with resultant death of animals.[11]

Results were processed statistically using Statistical 11.0 for Windows with a calculation of the mean and its standard deviation. Accuracy of intergroup variances was assessed using Student's t-test (under normal distribution), nonparametric Mann-Whitney U test, Fisher's angular transformation (in case of accounting in alternative form: survival-death). Variances were considered significant with a $p < 0.05$.

RESULTS

In previous studies, we used isopropanol as a solvent for recrystallization of obtained compounds.[4] This solvent is acceptable for use in the commercial scale synthesis of API; it is categorized as Toxicity Class III, acceptable residual level being 0.5% (according to the State Pharmacopoeia of Ukraine).[12]

Compounds 1-3 are insoluble in water. Since recrystallization conditions may influence further solubility and wettability of compounds, we decided to study the effect of solvent for recrystallization on physicochemical and pharmacological properties of substances in addition to isopropanol, also used for purification as solvents were ethanol and its aqueous solution (2:1). Losses of the compounds during recrystallization from ethanol are seen to be significantly higher than with isopropanol (Table 1). When a water and ethanol mixture (1:2) was used as a solvent loss of the compounds were comparable to those observed at crystallization from isopropanol (Table 1).

Table 1: Substances yield after recrystallization from different solvents.

Solvent	Substance yield, %					
	Compound 1	Code	Compound 2	Code	Compound 3	Code
Isopropanol	92	1.1	94	2.1	91	3.1
Ethanol-water (2:1)	94	1.2	94	2.2	93	3.2
Anhydrous ethanol	89	1.3	84	2.3	83	3.3

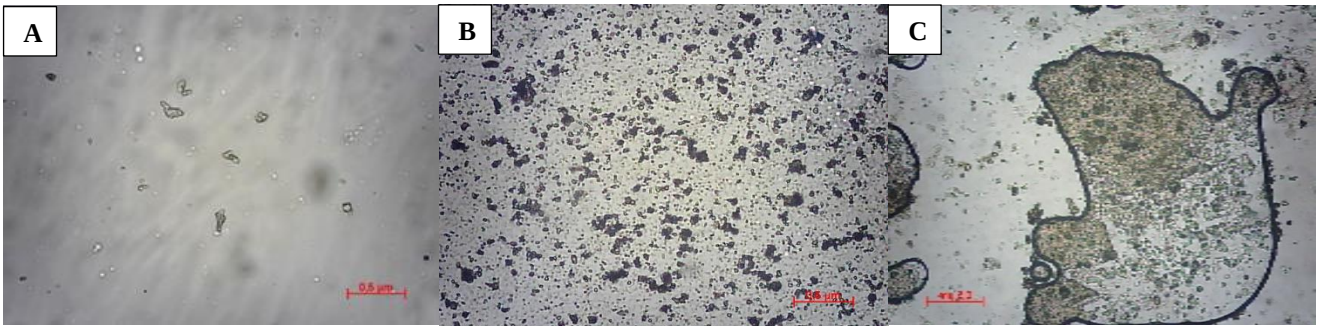


Figure 2: Microscopic study of compounds A-C in water.

Microscopic study of solubility of compounds 1.1-3.3 in water and Tween was the first stage of evaluating physicochemical properties of compounds recrystallized from different solvents. In view of yield reduction, no compounds recrystallized from anhydrous ethanol (2.3, 3.3) were studied in further experiments, except for compound 1.3, which yield does not depend significantly on the solvent for recrystallization (Table 1).

The results obtained showed the replacement of solvent for crystallization to be accompanied with slight changes in the dissolution rates in water (Figure 2 and 3) and Tween (Figure 4 and 5).

Thus, compound A is partially water soluble, B - very slightly soluble, C - practically insoluble. According to compound dissolution the solvents may be ranked as follows: isopropanol >ethanol-water >ethanol. For sample A a partial wetting with water is observed, but samples B and C are not wetted with water (Figure 2).

Figure 3 shows that replacement of solvent for recrystallization does not change solubility and wetting of compounds A and B in water. Both compounds are poorly wetted in water and are practically insoluble.

Microscopic study of dissolution of compounds A-C in Tween (Figure 5) demonstrated the following dependence: A - semi-soluble, B - practically insoluble, C- slightly soluble (A >B >C). At the same time, a reduction in solubility is observed in the following ranking: A >B >C.

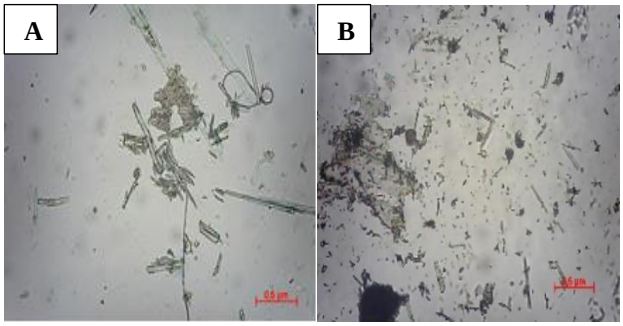


Figure 3: Microscopy of substances A-B in water.

Similar results are observed for compounds A, B - They are practically insoluble in water and not wetted in water (Figure 4).

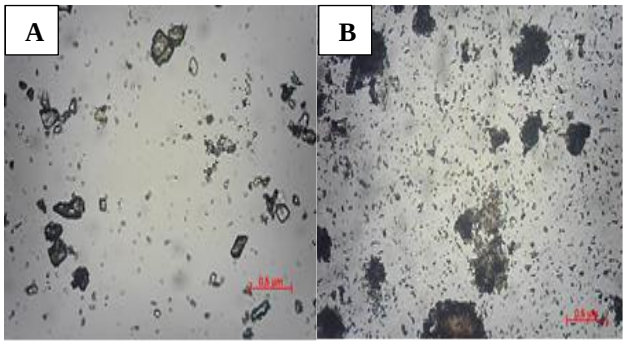


Figure 4: Microscopy of compounds A-B in water.

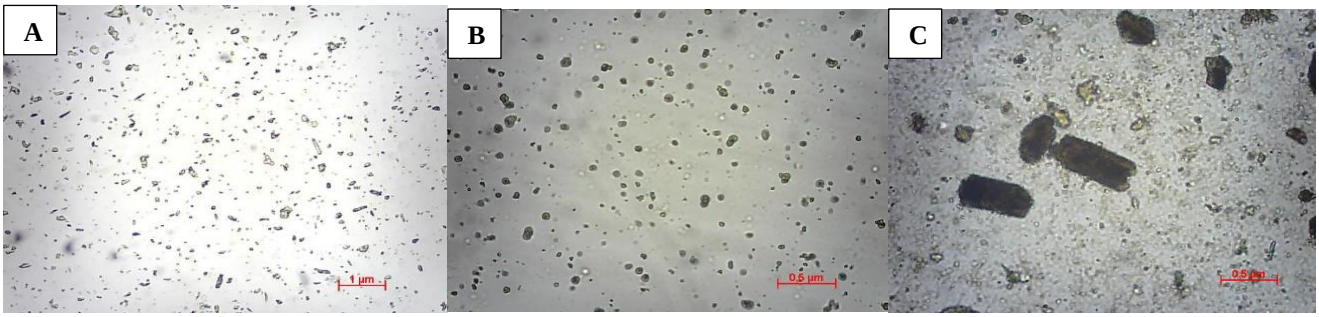


Figure 5: Microscopy of compounds A-C in Tween.

Experimental study of dissolution and wetting in Tween shows that a type of solvent for recrystallization does not practically affect the dissolution of compounds A and B in Tween: both compounds are practically insoluble, but a compound recrystallized from isopropanol (A) was wetted better with Tween compared to compound B.

Like in water, compounds A, B are practically insoluble in Tween, but sample B is wetted better with Tween compared to sample A (Figure 7).

Microscopic determination of the form and size of particles of substances obtained was the next step of study. The results obtained showed a difference in crystallographic characteristics depending on the type of solvent used for recrystallization.

Sample A had particles of granular form with smooth surface, with size of 0.01-0.4 µm (Figure 8). Compound B had particle size of 0.01-0.5 µm with rough surface, the inclusions of unidentified form were observed. Powder C being more homogenous; particle size of 0.01-0.2µm, granular form with rough surface.

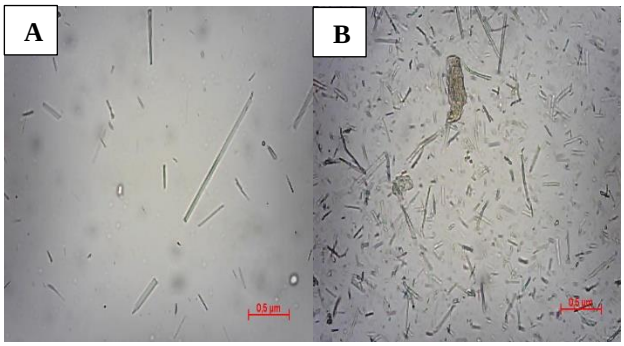


Figure 6: Microscopy of compounds A, B in Tween.

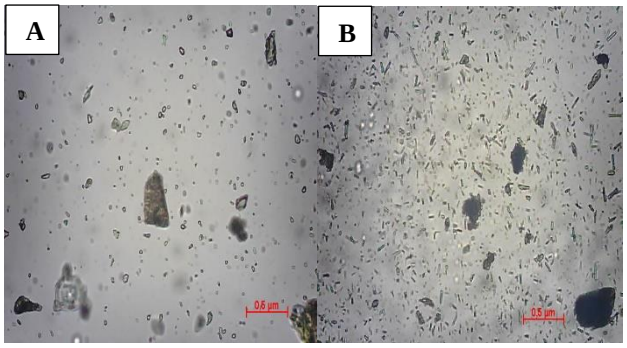


Figure 7: Microscopy of compounds A, B in Tween.

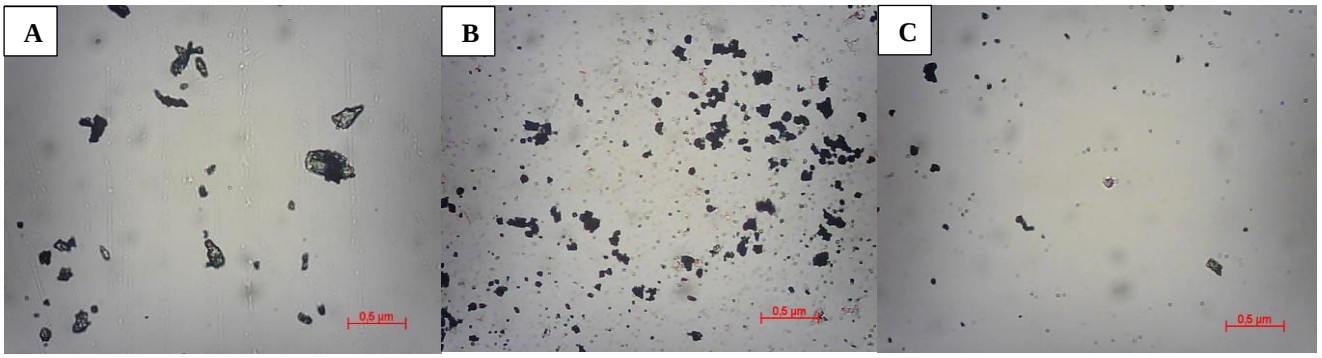


Figure 8: Microscopy of dry substances A-C.

Powder 2.1 was a fine-dispersed powder with particles size of 0.01-1.0 µm; crystals like smooth needles with inclusions of unidentified form (Figure 9). Powder 2.2 was a polydispersed powder with particles size of 0.01-0.7µm; needle-like form with smooth surface, with inclusions of unidentified form. Sample A was more homogenous by fractional content vs. sample B with inclusions, which differ in the form and size from general mass of crystals.

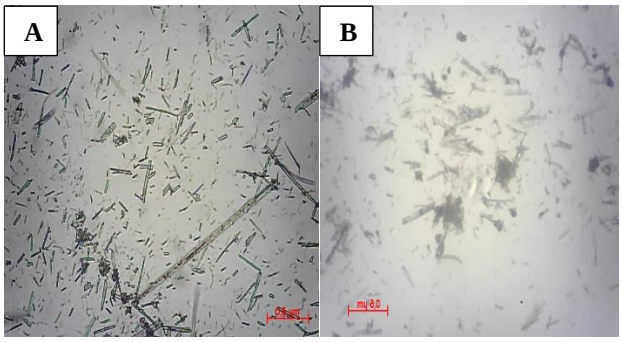


Figure 9: Microscopy of dry substances A-B.

Samples A, B were found to be polydispersed with particle size of 0.01-0.7µm; needle-like smooth form with inclusions of unidentified form (Figure 10).

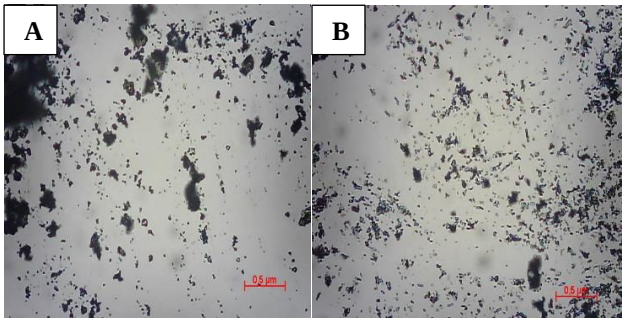


Figure 10: Microscopy of dry substances A, B.

Anticonvulsant effect of compounds crystallized from isopropanol (1.1, 2.1, 3.1) was studied in micronized form (a) - particle size up to 0.1 µm. Table 2 shows strong anticonvulsive effect demonstrated by all substances. It consists in reducing the main integral index of anticonvulsant activity (lethality): statistically significant by 3.6-fold ($p<0.05$) for compounds 1.1a, 3.1a and at the level of tendency by almost 2-fold ($p>0.05$) for compound 2.1a. In addition, compound 1. 1a significantly relieved the course of seizures by all parameters, prolonging the latency period (4.7-fold), reducing the number of mice with clonic seizures (by 16.7%) and with tonic seizures (by 43.2%). For compound 2.1a only decrease in the number of mice with clonic seizures was

found to be significant (by 16.7%). There was a tendency to increase in the period before initial seizures (due to the high data variability the difference from control did not reach the level of statistical significance) and decrease in the number of mice with tonic seizures. Sodium valproate as comparator has protected animals from death, but produced no significant effect on the course of seizures.

Table 2: Anticonvulsant effect of derivatives of 5-R-1-aryl-1,5-dihydro -4H-pyrazolo[3,4-d]pyrimidine-4-one 1-3.1 (a - dispersion up to 0.1 µm).

Group of animals, compounds	n	Dose, mg/kg	Latency period, min	% of mice with seizures		Lethality, %
				Clonic	Tonic	
Control	17	-	5.24±1.74	100	76.5	58.8
Sodium valproate	6	300	7.62±0.98	100	66.7	0**
1.1a	6	100	24.71±8.90*	83.3*	33.3*	16.7*
2.1a	6	50	14.13±9.12	83.3*	50.0	33.3
3.1a	6	100	5.06±1.09	100	66.7	16.7*

Statistically significant differences related to control: * - p≤0.05; ** - p<0.01

Table 3 shows that anticonvulsant effect of compounds 1-3 that were recrystallized from ethanol-water mixture (1.2. 2.2, 3.2) and ethanol (1.3) in micronized form up to 0.1 µm (a) and native form (b).

The most dependent on degree of grinding was found to be compound 3.2, which in the micronized form (3.2a) significantly prolonged the latency period by 2.8-fold (by 1.5-fold for 3.2b), reduced the number of mice with tonic seizures by 40% (3.2b-0%) and mortality by 63.3% (3.2b - increased mortality by 9.6%) against control.

Compound 2.2a (micronized) demonstrated the potent anticonvulsant effect, characterized by statistically significant 5-fold increase of the latency period and decrease in the number of mice with tonic seizures by 40% against control; the percentage of mice with clonic seizures and mortality had a tendency to decrease. The compound 2.2b demonstrated insignificant increase of lethality by 16.7% and latent period (1.48-fold) against control, as well as increase by 40% (p <0.05) of lethality and the number of mice with tonic paroxysms vs. compound 2.2a.

The tendency was observed related to 3.5-fold decrease of the latent period and increase in the number of mice with clonic seizures by 20%.

The effect of grinding degree of compound 1, crystallized from different solvents (1.2, 1.3), to the development of anticonvulsant activity differs. Dispersion of substance 1.2 does not influence the anticonvulsant activity, whereas substance 1.3a significantly better (by 60%) protects animals from death and relieves the course of seizures (decreases the number of mice with clonic and tonic seizures by 20%, prolongs (by 3.7-fold) latency period) vs. compound 1.3b.

Table 3: Anticonvulsant activity of derivatives of 1-aryl-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidine-4-ones 1.2-3.3 a, b depending on the degree of dispersion.

Group of animals, compounds	n	Dose, mg/kg	Latent period, min	Mice with seizures, %		Mortality, %
				Clonic	Tonic	
Control	6	–	3.09±0.22	100	100	83,3
Sodium valproate	6	300	19.73±8.24**	83.3	33.3**	16.7**
1.2a	5	100	7.12±4.08	100	100	80
1.2b	7	100	4.75±0.37**	100	100	100
1.3a	5	100	14.87±11.29	80	80	40##
1.3b	7	100	3.94±0.48	100	100	100
2.2a	5	100	15.94±11.04*	80	60*##	60##
2.2b	7	100	4.59±0.89	100	100	100
3.2a	5	100	8.75±3.17*	100	60*##	20*##
3.2b	14	100	4.50±0.92	100	100	92,9

1. Statistically significant differences vs. control: * - p≤0.05; ** - p<0.01.
2. Statistically significant differences of grinded vs. nongrinded compound: ## - p<0.01.

DISCUSSION

Losses of the compounds during recrystallization from ethanol are seen to be significantly higher than with isopropanol, which may account for higher dissolution of substances in ethanol. When a water and ethanol mixture (1:2) was used as a solvent loss of the compounds were comparable to those observed at crystallization from isopropanol. In addition, this permits to reduce the cost of finished products and to use less toxic solvent.

At this stage of study the purification (recrystallization) of compounds 1-3 by isopropanol and mixture of ethanol and water (2:1) is believed to be more appropriate for use in commercial-scale synthesis in terms of their yield.

Taking into account potential changes in the physicochemical characteristics, particularly solubility, and in related pharmacological properties, compared were physicochemical and pharmacological properties of compounds recrystallized from different solvents.

Thus, poor solubility in water and tween was a common regularity same as wetting substances 1-3 with these solvents when isopropanol as a solvent for recrystallization was replaced by ethanol-water mixture (2:1) and anhydrous ethanol (1.3). Pre-reduced hydrophily is an advantage for substances, which have to influence upon CNS activity.

Since pilot studies confirmed the difference in physical and chemical properties of substances, which may influence their bioavailability and pharmacological properties, substances 1.1-3.2. were pharmacologically screened for anticonvulsant activity to finally substantiate the choice of solvent for recrystallization.

Apart from crystal properties of substance its bioavailability is known to be influenced by particle size. According to the dissolution equation, an increase in total area of substance surface contacting liquid medium of GIT, contributes to the increase of solubility.[13] The less particle size is, the more area of active surface, dissolution rate and bioavailability are. This fact is especially important for poorly soluble medicinal products. Since the investigated compounds 1-3 are the case, particularly when recrystallized from ethanol-water mixture (1.2. 2.2, 3.2) and ethanol (1.3), it was decided to investigate and compare their anticonvulsant activity in micronized form up to 0.1 μm (a) and native form (b).

Table 3 shows that anticonvulsant effect is expected to depend considerably on both purification and degree of grinding of compounds. The most dependent on degree of grinding was found to be compound 3.2. Also, anticonvulsant effect of compounds crystallized from isopropanol (1.1, 2.1, 3.1) in micronized form (particle size up to 0.1μm) was the strongest.

Summarizing the effect of biopharmaceutical aspects on expressiveness of anticonvulsant activity, it should be emphasized that there is a significant dependence of the latter on compound's dispersion - the smaller the size of crystals the higher anticonvulsant activity. It can be explained by fast dissolution of fine-dispersed substances, thus increasing the bioavailability of compounds under study.

CONCLUSION

The effect of solvent on recrystallization of derivatives of 1-aryl-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidine-4-ones and degree of dispersion of substances on development of anticonvulsant activity were studied. The results obtained revealed the optimal solvent for recrystallization of compounds 1-3 to be isopropanol: compounds, purified by recrystallization from isopropanol, had higher solubility in water and tween; also, they had a tendency to increase anticonvulsant activity.

The differences were established in crystallographic characteristics using different solvents for recrystallization, but no dependence of anticonvulsant activity on compound crystal form 1.1-3.3 was revealed.

The dependence of anticonvulsant activity of compounds 1-3 on the degree of dispersion was proved: the smaller particle sizes the higher anticonvulsant activity. The optimal degree of dispersion is particle size of up to 0.1μm.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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