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STUDY OF ACUTE TOXICITY OF A NEW VETERINARY DRUG FOR INTTRAMMARY INTRODUCTION

Zhanna Polova
Department of pharmaceutical and industrial technology of medicines
O. O. Bogomolets National Medical University
13 T. Shevchenko blvd., Kyiv, Ukraine, 01601
zpolova@ukr.net

Natalia Seredinskaya
Laboratory of pharmacology of effector organs and systems
Institute of Pharmacology and Toxicology NAMS of Ukraine
14 Anton Cedic str., Kyiv, Ukraine, 03127
nvivalna@gmail.com

Abstract

Preclinical studies of veterinary medicinal products are important and compulsory studies in the development of new dosage forms. The aim of preclinical research is to determine the toxic effect and therapeutic efficacy of the test substance-the future dosage form, its effect on the body's basic systems, as well as the identification of possible side effects.

This work is part of the research on the development of the composition and technology of the veterinary drug – a solution for intramammary application, conventionally called "Argocide", intended for the treatment of mastitis in cattle.

A study of the acute toxicity of the intramammary veterinary drug was carried out in experiments on white rats of both sexes, according to the requirements for potential medicines. The establishment of the value of the average lethal dose (LD50) of the veterinary drug "Argocide" with intramuscular single administration to white mature rats is impossible due to the absence of animal death even when the drug is administered at doses exceeding 5.0 ml/kg. This experiment allows the veterinary preparation "Argocide" to be classified as practically non-toxic compounds (V class).

The analysis of the results of the conducted studies indicates the relative harmlessness of the potential drug for veterinary medicine and allows us to foresee that the "Argocide" preparation can be classified as low-risk substances, which justifies the expediency of its further study and introduction into practice.

Keywords: preclinical studies, acute toxicity, intramammary veterinary drug, "Argocide".

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1. Introduction

Preclinical studies of veterinary medicinal products are important and compulsory studies in the development of new dosage forms, especially in the context of the implementation of the legislation on the control of chemical compounds adopted by member countries of the Organization for Economic Co-operation and Development. First of all, this concerns the implementation of a scrupulous assessment of the potential hazard in conditions of guaranteed quality [1]. The aim of preclinical research is to determine the toxic effect and therapeutic efficacy of the test substance – the future dosage form, its effect on the body's basic systems, as well as the identification of possible side effects. The implementation of the rules of GLP system fully guarantees the quality of innovative medicinal veterinary drugs, their high therapeutic effectiveness [2, 3].

At the initial stage, primary toxicological studies and pharmacological evaluation of new active pharmaceutical ingredients, their separate components, various forms of new veterinary preparations are carried out, which not only determine the successful development of further experimental, clinical research and practical developments, but also has a decisive influence on the possibility of creating a highly efficient competitive and a low-toxic drug. Meeting the requirements of GLP provides a rationale for the safety of pharmaceutical development of a veterinary drug [4].

This work is a continuation of the research on the development of the composition and technology of a veterinary drug – a solution for intramammary administration under the conventional name "Argocide" [5, 6]. The developed combined medicinal product is intended for the treatment of subclinical inflammation of the mammary gland (mastitis) in agricultural animals, in particular in cattle [7].

Mastitis is an inflammation of the mammary gland that occurs in response to the influence of unfavourable environmental factors, in conditions of a decrease in the resistance of the organism and the complication of the infection. Inflammation of the mammary gland leads to a decrease in milk production, changes in the chemical composition, physical and biochemical properties of milk, as a result of which it loses nutritional value, technological properties, which affects its quality and safety [8]. The course and consequences of mastitis depend not only on the localization of the process and the virulence of the pathogen, but also on the immunobiological status of the whole animal's organism and the reactivity of the breast tissue [9, 10]. The development of the inflammatory process in the mammary gland occurs as a result of the action of mechanical, physical, chemical and biological factors. In particular, the biological factor accounts for 85 % of all cases of mastitis [11].

The spread of mastitis is explained by physiological loads on the organism of highly productive cows, inaccuracies in keeping and feeding, as well as non-observance of veterinary-sanitary requirements. To date, preparations based on antibiotics remain the main group for treating patients with mastitis of animals [12, 13]. However, the massive use of antibiotics for the treatment of mastitis has revealed a number of negative factors: the emergence of antibiotic-resistant strains of microorganisms; development of dysbacteriosis and decreased immune status; the presence of antibiotics in milk after the termination of the course of antibiotic therapy and as a consequence, the emergence of unwanted reactions in people who consume such milk [14]. One solution to the problem of dairy cattle disease is the pharmaceutical development of innovative veterinary drugs with antimicrobial substances that do not cause resistance.

2. Aim of the research

Study of acute toxicity of test specimens of a combined veterinary preparation under the conventional name "Argocide", a solution for intracisternal administration, with intramuscular injection of mature white rats.

3. Materials and methods

The research was carried out at the Institute of Pharmacology and Toxicology of the National Academy of Medical Sciences of Ukraine. Experiments in which animals were used was conducted in accordance with the international requirements for the humane treatment of animals and compliance with the requirements of Directive 86/609/EEC regarding the protection of animals [15, 16]. All experimental animals were kept in standard sanitary conditions. During the experiment, the animals were kept in a vivarium at 20–24 °C, humidity 30–70 % natural light mode "day-night", standard cells, with a balanced diet.

Investigation of acute toxicity of intramammary veterinary preparation was carried out in experiments on white non adult rats of both sexes (24–12 each sex; 2 animals at each of the investigated doses; 6 control animals (3 of each sex), according to the requirements of potential drug [1, 17]. The drug in the dosage form of solution for intracisternal administration was administered at varying doses – from 2.0 to 6.3 ml/kg at intervals – once daily [18] The volume of liquid which is injected animals. did not exceed volumes defined by the rules of pre-clinical research of drugs [2] The separate group of animals (control) they were treated with solvent (vehicle) preparation – water for injection, in a volume of 1.5 ml intramuscularly.

The duration of observation of the state of the animals after a single administration of "Argocide" in a drug form for intracisternal administration was 14 days. Observation of clinical signs of toxicity and death of the animals was carried out on the first day after administration of the veterinary drug continuously for one hour, and then at 2, 3, 5 hours after

administration. Over the next 14 days, each animal was observed daily, twice a day in 10^{00} and in 17^{00}. We registered cases of appearance and/or disappearance of clinical signs of toxicity, including – animal deaths.

The individual body weight of the animals was recorded before 10^{00} before the administration of the drug, and then 3, 7 and 14 days after the administration of the drug "Argocide". Changes in body weight were calculated in comparison with the weight on the day of administration and the body weight of the animals in the control groups.

Macroscopy. 14 days after the administration of the drug, all animals were withdrawn from the experiment by euthanasia by cervical dislocation under light ether anesthesia. All animals were subject to complete external examination, autopsy taking into account registered clinical disorders and detailed examination of internal organs. Before euthanasia at the end of the experiment, all animals were starving for 3 hours. It was planned to conduct a macroscopic study of animals that may die, immediately after the fact of death was established or the next morning (in 11^{00}), if the animal died at night.

Weight of organs. The following organs were weighed: liver, kidneys, spleen, lungs, and heart. Paired organs were weighed together. The relative mass of organs was calculated in g per 100 g of body weight.

Statistical processing of data. The data are given as mean ± mean error (M±m). Statistical processing of data was performed using of the Student t-test. The changes were considered statistically significant at $p<0.05$. For the statistical processing of data, the MS Excel program was used [19, 20].

5. Results of the research

The degree of acute toxicity of the drug is characterized by a dose value, one-time (or multiple for 1 day) administration of which causes the death of 50 % of animals (LD_{50}).

The main criterion for the toxic effect of the drug was the death of animals. At the same time, data on possible dose-dependent effects of the drug on various body functions were recorded in animals for 14 days, namely: the individual body weight of animals, general condition, changes in body position, skin condition, color of mucous membranes, body temperature (rectal), presence/lack of miosis/mydriasis, lacrimation, salivation, rhinorrhea, changes in the color of urine and feces and their frequency, drowsiness, tremors, convulsions, piloerection, cardiovascular, central nervous and respiratory activity system.

The results of changes in body mass and body temperature (M±m) in sexually mature white rats after a single intramuscular injection of the "Argocide" preparation are presented in **Table 1**.

Table 1

The dynamics of mass and body temperature (M ± m) in mature white rats after a single intramuscular injection of the drug "Argocide" in a dose of 6.3 ml/kg.

Group	Time of observation, (days)			
	Initial data	3 days	7 days	14 days
Control, weight, g (n=6)	182.6±1.08	187.3±1.60	192.8±1.95*	197.1±1.13*
Control, temperature, ºC (n=6)	38.1±0.14	37.9±0.16	37.9±0.13	38.0±0.14
Experiment, weight, g (n=24)	181.2±2.2	190.2±2.45	195.1±1.95*	199.8±2.4*
Experiment, temperature, ºC (n=6)	38.1±0.11	38.4±0.12	38.4±0.12	38.2±0.10

*Note: * – p <0.05 relative to the original data*

When administered intramuscularly to white rats, the drug "Argocide" in a dosage form is a solution for intracisternal administration (applied at doses of 3.16 ml/kg body weight of the animal and higher), the rats displayed certain clinical signs of intoxication, namely: cell congestion, decreased motor activity and reactions to external stimuli, which were noted immediately after the administration of the drug, as well as during the day of observation. Slowing down movements and violation of coordination of movements (ataxia) were noted during the first two hours after a single injection of the drug.

More active motor reactions were observed only 3–5 hours after the administration of the drug, ataxia was no longer noted at this time, while extension of the hind limbs during movement in the cage was observed simultaneously within 1 day after the introduction of the "Argocide". By the third day after the administration of the preparation, the animals had an untidy appearance, which was exuded in appearance – a damp coat, but the consumption of food and water was usual. Other signs of intoxication – seizures, tremors, salivation, changes in respiratory rate, cyanosis of the visible mucous membranes, blepharospasm, lateral position, etc. – in animals were not noted.

After 3 days after intramuscular administration of the drug "Argocide", no clinical signs of intoxication were observed: animals willingly consumed food and water, reactions to external stimuli were common, animals actively moved, a neat appearance were even after administration of sufficiently high doses of the drug – 5.0 ml/kg and 6.3 ml/kg of body weight of the animal.

In animals receiving the drug in low doses (2.0 and 2.5 ml/kg), clinical signs of intoxication were not observed at all. No cases of death of rats were noted, as evidenced by the data presented in **Table 2**.

Table 2

Acute toxicity study of "Argocide" preparation in experiments on white mature rats with intramuscular injection

Animal sex	Doses of the Argocide, ml/kg					
	2.0	**2.5**	**3.16**	**3.98**	**5.0**	**6.3**
Female	0/2*	0/2	0/2	0/2	0/2	0/2
Male	0/2	0/2	0/2	0/2	0/2	0/2

*Note: * – the ratio of the number of dead animals to the number of animals in the group by the indicated dose of the drug*

Among the animals of the control group, after intramuscular introduction of "Argocide" death was not recorded, their behavioral and vegetative functions are unchanged. All control animals (n=6) were active, had smooth hair and clean skin.

Weight of internal organs. A study was made of the absolute mass of the internal organs of sexually mature rats, which are euthanized 14 days after a single injection of the study drug, as well as calculations of the relative mass of internal organs in g per 100 g of body weight of the animal. The absolute mass of the internal organs in the mature rats of the experimental groups did not undergo actual changes in relative to the corresponding index in the rats of the control group. The rats of the control group were examined for comparison with the rats of the test group at the corresponding (identical) observation times. This allowed to provide averaged data for all organs in animals receiving different doses of the drug. The results are shown in **Table 3**.

The relative mass of the internal organs in the mature rats of the experiment groups in obtaining the highest studied doses (3.98, 5.0, and 6.3 ml/kg) did not differ significantly from the relative mass of the internal organs of the rats in the control group. The results are shown in **Table 4**.

Macroscopic studies. Pathomorphological examination of organs and tissues of white mature rats of both sexes was conducted, which survived and was euthanized after 14 days of observation. The animals were once administered the drug "Argocide" in a dosage form solution for intracisternal administration in various doses.

Table 3

Absolute mass (M±m, g) of the internal organs of mature white rats subjected to euthanasia 14 days after a single intramuscular injection of the preparation "Argocide" in a dose of 6.3 ml/kg

Organs	Experiment-female (n=6)	Control-female (n=3)	Experiment-male (n=6)	Control-male (n=3)
Heart	0.89±0.01	0.90±0.01	0.87±0.01	0.90±0.01
Lungs	1.31±0.02	1.29±0.2	1.20±0.2	1.24±0.1
Liver	10.54±1.30	10.34±1.1	10.13±1.2	9.86±0.8
Spleen	1.27±0.02	1.19±0.03	1.18±0.03	1.19±0.3
Kidneys	2.29±0.07	2.16±0.1	2.14±0.08	2.15±0.09

Table 4

The relative mass of internal organs (g/100 g of body weight M±m) in white rats (n=6) after a single intramuscular injection of the preparation "Argocide" at a dose of 6.3 ml/kg, and rats of the control group (n=6), who were euthanized

Organs	Experiment-female+male (n=6)	Control-female (n=3)	Control-male (n=3)
Heart	0.46±0.01	0.45±0.01	0.45± 0.01
Lungs	0.66± 0.03	0.66±0.03	0.63±0.02
Liver	5.35±0.15	5.25±0.2	5.01± 0.24
Spleen	0.65±0.01	0.61±0.01	0.62±0.02
Kidneys	1.17±0.03	1.10±0.01	1.10±0.04

According to the external examination of animals, no signs of pathological changes in their condition were found, hair and skin were clean, the subcutaneous layer of fatty tissue was moderately expressed, no mucous membranes and skin lesions were observed. Eyes, nose, lips, mouth, anal opening and external genital organs had a normal structure in all animals of experimental groups.

When the sexually mature animals were autopsied, the serous membranes of the abdominal, pleural and pericardial cavity were smooth, shiny, without signs of damage or inflammation, the amount of free serous fluid in the cavities is not increased. The brain, organs of the abdominal cavity, as well as the small pelvis are located without deviations from the norm, the colour and consistency of them were not changed; organs of the chest cavity (lungs, heart) had the usual colour and blood filling. Myocardium had viscoelasticity consistency in all animals of all groups, homogeneous on the cut, dark red (cherry) colour. Endocardium of the atria and ventricles was smooth, shiny, the trabecular relief of the ventricular cavity is clear. Heart valves were thin, elastic. Aortic wall of usual thickness, elastic, with a brilliant intima of a whitish colour. In animals that were euthanized, lung tissue is not uniform, loose consistency.

The surface of the mucosa of the larynx, trachea and large bronchi was not changed, covered with a small amount of mucus, the lumen of these organs was free.

In the gastrointestinal tract of all rats of the experimental and control groups were observed no alternative changes or ulcers. The mucous membrane of the stomach had pronounced crypts, without swelling, without damage to the erosive or ulcerative nature.

The liver was of ordinary size, its capsule was smooth, shiny, the liver tissue was uniform, dense-elastic, in a section of reddish-red color. The pancreas was located in the mesentery of the duodenum, a lobate structure, its tissue was whitish-yellow, with no signs of damage and inflammation.

Kidneys were of usual shape and size. Their surface was covered with an unaltered dense fibrous capsule, which was easily separated from the parenchyma of the organ. The tissue of the kidneys was of elastic consistency, with no signs of structural changes, the cortical and cerebral layers of the organ were clearly defined on the cut. Bowls are not dilated, their mucous membranes are thin, smooth, shiny, pale pink.

The wall of the bladder is thin, elastic, pale pink in the cavity of a small amount of transparent urine.

The state of endocrine organs of rats – thyroid gland, adrenal glands had no visible deviations from the norm. Adrenals were of usual size and shape, on a cut of yellow colour. Thyroid gland of usual size and shape, lobate structure, on a section of brownish-red colour. The macroscopic structure of the thymus and spleen was the same as that of the control animals, with no signs of alternative changes, hemocirculatory disorders of atrophy and hypertrophy.

6. Discussion

Pharmacological and toxicological results of studies of drugs and compounds of silver are widely represented in the scientific literature. The authors [21] give data on the toxic properties of Argumistin veterinary medicinal product with a mass content of colloidal silver of 10 and 50 μg/ml, in a dosage form a solution for local and internal use. It has been established that Argumistin is a low-risk chemical substance after introduction into the stomach by laboratory animals according to the classification of the average lethal dose. With prolonged enteral administration, preparations at doses of 5 ml/kg did not exert any noticeable inhibitory effect on the overall state of white non-linear mice.

N. N. Shkil and co-authors determined the parameters of subchronic toxicity of the silver-containing drug Argovit on laboratory animals [22]. For white mice, the cumulation coefficient was 24.15, for white rats – 16.1, which made it possible to classify the test drug as non-cumulative. With the prolonged administration of increasing toxic doses of Argovit to white rats, they found no significant changes in the immunobiochemical parameters of blood serum. During the entire study period, no mortality of laboratory animals was detected, as well as violations of the function of their gastrointestinal tract.

N. S. Ponomar with co-authors studied the acute toxicity of a new drug of ionized silver. The obtained results testified to the absence of any influence of even the maximum possible doses of the study drug on behavioral, neuromuscular and vegetative reactions in rats, as well as the lethality of animals in both enteral and parenteral administration. LD_{50} of a silver preparation in this connection was not established, and the authors of the study drug are classified as low-toxic and safe [23].

A dose-dependent assessment of the toxicity of silver nanoparticles in Wistar rats in vivo is presented by D. K. Tiwari, T. Jin, J. Behari. The studies are devoted to the study of the effect of various doses of silver nanoparticles on rats. Four different doses (4, 10, 20 and 40 mg/kg) were administered intravenously. It has been shown that silver nanoparticles in doses (<10 mg/kg) are safe for biomedical applications and have no side effects, but at high doses (>20mg/kg) are toxic [24].

Ukrainian scientists have studied the acute toxicity of silver nanoparticles in a colloidal solution. It was found that LD_{50}, when administered intraperitoneally to male and female BALB mice, was 34.53±3.87 mg/kg and 22.17±2.36 mg/kg, respectively. The authors classify

silver nanoparticles as substances of the third toxicity class "moderately toxic compounds" according to the K. K. Sidorov classification (1973), and LD_{50} when administered intravenously to white non-linear mice for males and females is 83.2±10.93 mg/kg and 99.92±11.71 mg/kg, respectively [25].

Park K., Park E. J., Chun I. K. et al. investigated the bioavailability and toxicokinetics of silver-coated nanoparticles coated with citrate. Male rats were administered orally or intravenously 1 or 10 mg/kg silver nanoparticles. It was found that, after oral administration, the concentration of silver nanoparticles in blood was very low. However, after injection of the tail vein, a high level of silver in the blood was detected. When silver nanoparticles were administered to rats at a dose of 1 mg/kg, the silver concentration in the blood was significantly increased 10 min after injection; the level subsequently decreased. In rats receiving silver nanoparticles at a dose of 10 mg/kg, an elevated level of concentration persisted during the experimental period. It is noted that silver nanoparticles accumulate in the liver, lungs and kidneys [26].

Souza L. R. R., da Silva V. S., Franchi L. P., de Souza T. A. J. pay attention to the need for in-depth study of the mechanism of cytotoxicity of some silver nanoparticles, as well as possible toxic effects [27].

As part of the pre-clinical study of the combined veterinary drug "Argocide" containing a silver compound, a study was conducted of the acute toxicity of the drug form, a solution for intracisternal administration. Doses were calculated for the dosage form, and not for the active pharmaceutical ingredient. It should be noted that such studies are being conducted for the first time.

According to the analysis of the data obtained (**Table 1**), it was determined that such integral indices of vital activity of animals as mass and body temperature did not experience significant individual fluctuations during the 14 days of observation after a single intramuscular injection of the drug "Argocide". At the same time, a physiological increase in body weight in animals was noted. The animals of the experimental and control groups gained weight in accordance with the physiological norm.

A study of the acute toxicity of the preparation "Argocide" in experiments on white mature rats with intramuscular introduction (**Table 2**) showed that among the animals of the control group, when intramuscular "Argocide" was administered, death was not recorded, their behavioural and vegetative functions were unchanged.

With external examination, autopsy and macroscopic examination of rats of both sexes who received Argocid once, the solution for intracisternal administration in different doses in the range from 2.0 ml/kg to 6.31 ml/kg of body weight, after 14 days of the introduction, there were no signs of a violation of hemocirculation in the tissues of the heart, lungs, stomach and small intestine.

In groups of control and experimental animals, no alternative, inflammatory and hemodynamic disorders in various organs and tissues were detected.

Thus, the research on determining the value of the average lethal dose (LD_{50}) of the veterinary drug "Argocide", a solution for intracisternal administration, with intramuscular single administration to white mature rats is impossible because of the absence of animal death even when the drug is administered in doses exceeding 5,0 ml/kg. The introduction of higher doses is considered inexpedient. This circumstance makes it possible to classify the preparation "Argocide", a solution for intracisternal administration, considering the classification of chemical substances according to the degree of danger, to practically non-toxic preparations (V class).

7. Conclusions

1. The acute toxicity of the intramammary veterinary drug under the conventional name "Argocide" was carried out, in the dosage form – a solution for intracisternal administration.

2. Tests were carried out on white non-linear sexually mature rats of both sexes with intramuscular injection in different doses – from 2.0 to 6.3 ml/kg.

3. It has been established that such integral indices of vital activity of animals as mass and body temperature did not experience significant individual fluctuations during the 14 days of observation after a single intramuscular injection of the "Argocide".

4. It was noted that after intramuscular administration of the preparation "Argocide" at doses of 3.16 ml/kg body weight of the animal and above, certain clinical signs of intoxication appeared in the rats. However, 3 days after the intramuscular application of the drug "Argocide", no clinical signs of intoxication were observed even with the administration of sufficiently high doses of the drug – 5.0 ml/kg and 6.3 ml/kg of body weight of the animal.

5. Animals receiving the drug in low doses (2.0 and 2.5 ml/kg) had no clinical signs of intoxication at all. No cases of death of rats were noted.

6. Conducting studies to establish the value of the average lethal dose (LD_{50}) of the veterinary drug "Argocide" with intramuscular single administration to white mature rats is impossible because of the absence of animal death even when the drug is used in doses exceeding 5.0 ml/kg.

7. This experiment allows us to refer veterinary drug "Argocide", a solution for intracisternal administration, considering the classification of chemical substances according to the degree of danger, to practically non-toxic preparations (V class).

8. Analysis of the results of the conducted studies indicates the relative harmlessness of the potential drug for veterinary medicine and allows to foresee that the "Argocide" can be classified as low-risk substances, which justifies the expediency of its further study and introduction into practice.

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