

# EXPERIMENTAL MODELS AND INSTRUMENTAL SURVEYS FOR RISK ASSESSMENT IN HYGIENE AND EPIDEMIOLOGY

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UDC 615.9:[577.175.1.08:631.811.98]:613.26

DOI: 10.21668/health.risk/2017.3.14.eng

## PECULIARITIES OF TOXIC EFFECTS EXERTED BY HEXYL ETHER OF 5-AMINOLEVULINIC ACID AND GROUNDS FOR WORKING OUT OUR REGULATIONS OF ITS SAFE PRODUCTION AND APPLICATION

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*Our research object was hexyl ether of 5-aminolevulinic acid applied as plants growth regulator which was synthesized with an original technology by Bioorganic Chemistry Institute of Belarus National Academy of science. Our research goal was to determine peculiarities of toxic effects exerted by this new plants protector on experimental models in vivo/in vitro and to give grounds for hygienic standards of its contents in various media which were to provide its safe production and application. We conducted our research with the use of toxicological, physiological, hematological, biochemical, immunologic, cytogenetic, cytological, and statistical methods. We were the first to perform toxicological assessment of this new plants growth regulator under different regimes, doses and ways of introduction into laboratory animals' bodies and it helped us to detect its toxicometric parameters and peculiarities of its biological effects which became apparent through skin-resorptive and cumulative properties, irritating impacts on mucous tunics, moderate reproductive toxicity without substantial signs of gonadotropic, mutagenic, and allergenic impacts on a body. We detected that toxic impacts exerted by the examined substance on a body was combined with membrane-tropic and cytotoxic effects. We determined criteria and limiting parameters of hazardous effects exerted by hexyl ether of 5-aminolevulinic acid in the course of a chronic experiment, and it gave us grounds for fixing allowable daily dose for a man and for working out a number of regulations on the substance contents in environmental objects (working area air, water, and soil), in food raw materials, and in food products (grain, rape, rape and linen oil). The obtained results were used as a basis for fixing 9 hygienic standards and were used for the state registration of this plant growth regulator; it will provide its safe production and application in agriculture.*

**Key words:** hexyl ether of 5-aminolevulinic acid, plants growth regulators, toxicity, danger, biological effects, laboratory animals, cells cultures, hygienic standards.

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Application of plants growth regulators is a very promising trend in agricultural production development as such regulators complement application of fertilizers and pesticides. Growth regulators are usually physiologically active substances, either natural or synthetic ones, which make for increase in crop production efficiency via influencing intensity and directivity of

In order to achieve new developments in the sphere, experts of Bioorganic Chemistry Institute of Belarus National Academy of Science created a new plant growth regulator on the basis of hexyl ether of 5-aminolevulinic acid (HE-ALA) [8] which efficiently stimulates growth and development of certain agricultures as well as increases plants resistance to unfavorable

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Ó Vlasenko E.K., 2017

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plants morphogenesis processes.

cultivating conditions [3,11,12].

To provide safe experimental-industrial synthesis and application of this new substance, it was necessary to accomplish its complete toxicological assessment together with justifying required standards for its contents in human environment objects. All the above stated proves that complex toxicological research aimed at assessing HE-ALA toxicity and danger was really vital; it was also necessary to detect its toxic dynamics and probable ways in which it could exert hazardous impacts on a body and to justify limiting hazard parameters.

**Our research goal** was to detect peculiarities of toxic effects exerted by HE-ALA on experimental models *in vivo/in vitro* and to give grounds for a set of hygienic standards providing its safe production and application.

**Research objects and methods.** Experimental research was performed on 200 non-linear white mice (17–23 g), 325 randomly bred white rats (180–210 g), and 3 white rabbits (4.2–4.5 kg), in full conformity with methodical guidelines [5,13,14].

When performing toxicological research we modeled acute, sub-acute and chronic intoxications. During acute experiments HE-ALA toxic parameters were determined at intragastric, epicutaneous, and inhalation introduction into a body with consequent calculation of lethal doses via probit analysis and determination of values showing potential acute intoxication danger. Ability to cumulate in sub-acute experiments at intragastric introduction and peculiarities of skin-resorptive effects were determined on white rats in repeatable 30-day experiments. Chronic experiment was conducted on white rats during which HE-ALA was intragastrically introduced into animals during 6 months in doses equal to 110, 30 and 11 mg/kg.

We studied sensitizing activity and irritating effects exerted by HE-ALA on eyes mucous tunics and intact cutaneous coverings in conformity with the Instruction No. 1.1.11–12–35–2004 [14].

HE-ALA influence on white rats' reproductive function was examined as per A.A. Dinerman technique [4]. We applied Ames test [17] and cytogenetic chromosome aberrations technique on lymphocytes from human peripheral blood to assess mutagenic activity [18]. HE-ALA cytotoxic properties were studied with transplantable cultures of human embryonic fibroblasts (HEF) and human lung adenocarcinoma A-549 cultivated as per L. Hayflick and P. S. Moorhead technique [19].

To assess the state of toxicity eliminating mono-oxygenase system, we applied microsomal function of white rats' liver [20], in which we determined P450 and P420 cytochromes content, P450-reductase activity, and kinetic parameters (Km, Vmax) of 7-Ethoxycoumarin и 7-Ethoxyresorufin oxidation reactions.

We examined membranes structural and functional properties with the use of pyrene fluorescent probe introduced into blood shadows suspension of white rats' erythrocytes under exposure to HE-ALA in *in vivo* and *in vitro* experiments [2].

All the research results were processed with conventional techniques. Discrepancies between focus groups and control groups were considered to be statistically significant at  $p < 0.05$ .

Results of toxicological and hygienic assessment of this new compound can in future be used for setting safety parameters as per permissible risk criteria.

**Results and discussion.** We determined basic quantitative toxicity parameters (DL16, DL50, DL84) and danger parameters (S, R) under a single intragastric

introduction of HE-ALA into white mice and rats, both male and female; these parameters didn't have any specific discrepancies (specific sensitivity coefficient is equal to 2.6) and sex discrepancies and allowed to consider the examined substance to be moderately dangerous (Table) [10].

Table

Toxicity and potential danger parameters of acute HE-ALA intoxication

Species	Lethal doses, mg/kg			Potential danger parameters of acute intoxication	
	DL <sub>16</sub>	DL <sub>50</sub>	DL <sub>84</sub>	S	R
Male mice	2540	3000 (2630÷3420)	3540	1,18 (0,99÷1,4)	1,39
Female mice	2470	3170 (2710÷3710)	3900	1,26 (1,13÷1,41)	1,58
Male rats	6350	8800 (6560÷11800)	12500	1,41 (0,97÷2,0)	1,97
Female rats	4800	7800 (5740÷10600)	12600	1,62 (1,12÷2,35)	2,63

Note:

1. DL<sub>16</sub>, DL<sub>50</sub>, DL<sub>84</sub> are doses leading to 16%, 50%, 84% of deaths.
2. S is a function of "dose - effect" straight line tilt angle.
3. R is lethal doses span.

When we modeled acute inhalation intoxication under maximum achievable HE-ALA concentration we deleted no signs of intoxication and didn't register any deaths; the same situation was under a single introduction of 50% solution on white rats skin in a dose equal to 800 mg/kg. The obtained results prove there is no acute intoxication danger under the above-mentioned exposure ways.

When 50 µl of 50% HE-ALA solution were instilled into a posterior conjunctival fornix of a rabbit's right eye, we observed abundant lacrimation, moderate reddening of conjunctiva vessels, eyelids edema, and blepharospasm, and it allowed us to consider HE-ALA to possess apparent irritating

effects. We didn't detect any signs of the substance irritating effects when it had a single contact with intact cutaneous coverings of white rats.

We didn't reveal any sensitizing properties of HE-ALA when studying it in experiments on white mice via modeling reproduction of delayed-type hypersensitivity.

When HE-ALA was intragastrically introduced into male white rats, occurrence of lethal cases at the level equal to 1/5 DL<sub>50</sub> allowed us to calculate cumulation coefficient which was equal to 1.6 and characterized the substance as a highly cumulative one. We registered the most apparent changes at intragastric HE-ALA introduction into white rats during 30 days in a dose equal to 880 mg/kg (1/10 DL<sub>50</sub>); these changes were statistically significant shifts in behavioral activity, increase in summational-threshold parameters (STP) by 78% and in relative mass coefficients (RMC) of internal organs, namely liver, heart, lungs, spleen, kidneys, and adrenals. As for hematologic parameters, we revealed decrease in hemoglobin concentration by 13% and oxihemoglobin by 14%; quantity of erythrocytes, by 15% hematorcit, by 12%, in comparison with the control groups. There were other signs of disorders in acid-base blood balance: authentic decrease in partial oxygen pressure by 24%, increase in partial carbon dioxide pressure by 14%, and increase in bicarbonate blood capacity by 13% against the control. We detected the following changes in white rats' blood serum: 1.5 times increased activity of gamma-glutamyltranspeptidase (GGT) ( $p < 0.05$ ), 2.3 times more active alanine aminotransferase (ALT) ( $p < 0.05$ ), 2.0 times increase in crude bilirubin content ( $p < 0.05$ ), 1.5 times increased C3 complement component and increase in immunoglobulin G by 28% ( $p < 0.05$ ) in comparison with the control group. Hyperenzymemias occurrence with

such pathophysiological grounds as damage to hepatocytes membranes indicates the substance can damage membranes.

Under subchronic intragastric HE-ALA introduction in a dose equal to 440 mg/kg (1/20 DL50) we observed changes in motor activity of experimental rats and increase in STP by 60% ( $p < 0.05$ ). When experiments were over, we registered increase in RMCs of experimental rats' heart, kidneys, and adrenals ( $p < 0.05$ ), decrease in hemoglobin concentration in peripheral blood by 9%, and 1.6 times increase in crude bilirubin level in comparison with the control group ( $p < 0.05$ ). When an introduced HE-ALA dose was reduced to 110 mg/kg (1/80 DL50) we detected only 1.3 times increase in crude bilirubin in white rats' blood serum against the control group ( $p < 0.05$ ).

As we studied skin-resorptive HE-ALA properties with the substance being introduced as 50% solution (864 mg/kg) in 30 consequent applications on white rats skin on their backs we didn't reveal any outer signs of intoxication and didn't detect any deaths. However we observed a 21.6% increase in ALT enzymatic activity ( $p < 0.05$ ) and 22% increase in urea content ( $p < 0.05$ ) in blood plasma while pH of urine decreased to 6.0 ( $p < 0.05$ ) in comparison with the control group. Epicutaneous impact exerted by 25% HE-ALA solution in a dose equal to 341 mg/kg led to a 29.6% increase in ALT activity in blood serum of white rats ( $p < 0.05$ ). When HE-ALA impact went down to 75 mg/kg (5% solution), we didn't reveal any changes in biochemical parameters.

Standardization of this new plants growth regulator requires determination of its influence on reproductive function and examination of its possible mutagenic impacts [5].

A single intragastric introduction of the substance into female white rats in a dose

equal to 1/2 DL50 (3,900 mg/kg) during periods of intensive organogenesis caused clinical signs of intoxication; however we didn't detect any embryos deaths or any signs of teratogenic impacts. Pre-implantation mortality in experimental rats was insignificant. Post-implantation mortality was completely absent both in focus and control groups. Impacts exerted by the substance on the 9th day of pregnancy turned out to be the most crucial for post-natal development; we detected such signs of it as physical retardation of infant rats (development slowed down by 13% as per body weight and by 5% as per body length) ( $p < 0.05$ ). The obtained data reveal that HE-ALA has moderate reproductive toxicity. Even when exerting multiple impacts during the whole pregnancy in a dose equal to 1/40 DL50 (195 mg/kg), the examined substance didn't have any embryo-trophic or teratogenic effects and didn't exert any negative influence on postnatal development of offspring.

When HE-ALA was sub-chronically introduced intragastrically into male white rats in a dose equal to 440 mg/kg (1/20 DL50), it had weak gonad-toxic effects as per morphofunctional parameters of gonads which became apparent through testicles RMC growing by 12% ( $p < 0.05$ ) and 1.6 times decrease of spermatozoons motion period ( $p < 0.05$ ) in comparison to control. When an introduced dose was reduced to 110 mg/kg, we didn't detect the above-mentioned changes.

So, embryo-trophic and gonad-trophic effects were registered only when massive doses of the substance were introduced; such doses led to intoxication of parents' bodies and it gave us grounds to consider such changes in reproductive system parameters as signs of general toxic impacts exerted by HE-ALA [9].

We didn't detect any potential mutagenic activity of HE-ALA in concentrations equal to 1 and 10  $\mu\text{g/ml}$  in Ames test performed on *S. typhimurium* TA 100 strain with metabolic activation and without it; the same was in a cytogenetic test performed on human peripheral lymphocytes in vitro under exposure equal to 0.05, 0.1 and 0.25  $\text{mg/ml}$  [1].

To reveal mechanisms of HE-ALA toxic effects we studied a state of detoxification mono-oxygenase system of white rats liver under chronic intragastric introduction in a dose equal to 440  $\text{mg/kg}$ . We detected authentic 1.4 times increase in specific content of P450 cytochrome and 1.5 times increase in specific activity of P450-reductase but maximum speed  $V_{\text{max}}$  and Michaelis-Menten constant  $K_m$  of 7-Ethoxycoumarin and 7-Ethoxyresorufinoxidative reactions didn't have any discrepancies with the control group. We should point out there was a 3.0 times increase in specific content of P420 cytochrome in experimental animals in comparison with the control groups ( $p < 0.05$ ), which can possibly indicate that the examined substance is able to destabilize endoplasmatic reticulum membranes.

The results of experiments performed in vitro on HEF and A-549 cultures revealed that HE-ALA in concentrations equal to  $2 \times 10^{-3} \text{ mol} \times \text{l}^{-1}$  caused a monolayer degeneration when cells separated from glass. When concentration was equal to  $2 \times 10^{-4} \text{ mol} \times \text{l}^{-1}$  the substance suppressed HEF cells proliferation 2.8 times, and A-549 cells, 6.3 times, in comparison with the control. We didn't observe any significant influence of the substance on the examined cultures proliferative activity in experiment with exposure to HE-ALA being equal to  $2 \times 10^{-5} \text{ mol} \times \text{l}^{-1}$ : a number of HEF cells increased 16 times during this experiment, and a-549 cells, 26 times in comparison with the initial level before the substance was introduced

and it corresponded to normal growth of these culture under in vitro conditions. The calculated value of average efficient concentration of CL50 cytotoxic effects amounted to  $1.14 \times 10^{-4}$  ( $1.08 \times 10^{-4} \div 1.21 \times 10^{-4}$ )  $\text{mol} \times \text{l}^{-1}$  for HEF cells culture and to  $0.5 \times 10^{-4}$  ( $0.48 \times 10^{-4} \div 0.54 \times 10^{-4}$ )  $\text{mol} \times \text{l}^{-1}$  for A-549 cells culture.

We studied influence exerted by the substance on structural and functional properties of lipid bilayer state within the range of its cytotoxic effects. This influence was examined in vitro via introduction of pyrene fluorescent probe into blood shadows suspension of intact rats erythrocytes. When HE-ALA was added in concentrations equal to  $2 \times 10^{-5}$ ,  $2 \times 10^{-4}$ ,  $2 \times 10^{-3} \text{ mol} \times \text{l}^{-1}$  it caused pyrene fluorescence suppression without any changes in excimerization coefficient: ratio between excimers Fe at  $\lambda_{\text{em}} 475$  nanometers and Fm at  $\lambda_{\text{em}} 373$  nanometers; it proved lipid bilayer degradation occurred.

Our examination of structural and functional parameters which erythrocytes membranes had in subchronic experiment in vivo on white rats revealed there was a decrease in lipid bilayer microviscosity and annular (protein) lipid viscosity which depended on impacts exerted by HE-ALA in a dose equal to 440  $\text{mg/kg}$ ; it was proven by excimerization coefficients growth but their polarity parameters remained within control values range. We examined intensity of radiationless transfer of electronic excitation energy from tryptophan remains on pyrene and revealed an authentic 38% decrease in this parameter ( $p < 0.05$ ), which was the evidence that protein molecules were less submerged into lipid bilayer.

Chronic intragastric HE-ALA introduction into white rats in doses equal to 110, 30 and 11  $\text{mg/kg}$  during 6 months didn't cause any clinical signs of intoxication and death. We observed some changes in experimental

animals' nervous system when a dose was 100 mg/kg, namely a 34% decrease in motor activity and summational -threshold parameter in comparison with control groups ( $p < 0.05$ ). Impacts exerted by HE-ALA didn't cause any changes in biochemical parameters of blood serum, including crude bilirubin levels which was a marker parameter of subchronic impacts. Urinary system disorders were proven by an authentic decrease in urine hydrogen ions quantity (pH 6.2), 4.3 times lower daily urine output, and 2.6 times lower creatinine clearance against control groups.

Chronic intragastric HE-ALA introduction into white rats in a dose equal to 30 mg/kg led only to an authentic 35% decrease in summational-threshold parameter and it allowed us to determine this impact as being a threshold of a chronic effect (Limchr). Therefore, a functional state of nervous system as per changes in summational-threshold parameter can be considered a limiting parameter of HE-ALA chronic general toxic effects.

Sings of the substance toxic effect didn't occur when a dose was 11 mg/kg (maximum non-effective one) and it allowed us to use this value to calculate HE-ALA permissible daily dose (PDD). Allowing for the substance having considerable cumulative properties, we decreased maximum non-effective dose 100 times (assurance coefficient) and it made PDD being equal to 0.11 mg/kg. Permissible daily introduction (PDI) into a human allowing for PDD and average body weight being 50 kg should not exceed 5.5 mg/day (a sum of all the substance introductions out of various media).

We calculated predictive values of tentatively safe impact exerted by HE-ALA content in working area air as per formulas applied for all the pesticide groups [16]. As we analyzed the obtained values we detect-

ed that the lowest calculated hygienic standard value was 0.8 mg/m<sup>3</sup>.

Tentatively safe HE-ALA content in the atmosphere was set at 0.01 mg/m<sup>3</sup> as per calculations [7] allowing for molecular weight, basic toxicity parameters and tentatively safe content in working area air. When tentatively safe content is equal to this value, 0.2 mg of HE-ALA can penetrate a human body with atmospheric air and it is equal to 3.6% of permissible daily introduction (PDI).

To predict tentatively permissible content in water, we applied equations which described correlations between this value, fixed toxicological parameters (DL50), standard contents in other environmental objects (working area air), and physical constants [6]. Minimal calculated tentatively permissible HE-ALA content value was equal to 0.1 mg/dm<sup>3</sup>. Results of organoleptic examinations of water which contained HE-ALA at a minimum calculated level revealed that there was no smell at 20 °C and 60 °C.

Tentatively permissible HE-ALA concentration in soil was calculated as per permissible daily dose value [15] and was equal to 0.3 mg/kg. Agrotechnical application of the substance in pre-sowing treatment of barley, rape, and fiber flax seeds in conformity with the recommended expenditure rates up to 3 g/g (up to 150 ml/hectare) is unlikely to cause the substance contents in oil being higher than the fixed tentatively permissible concentration.

As the substance is to be applied in cultivation of spring barley, winter rape, and fiber flax, we performed calculations to give grounds for maximum permissible quantities of HE-ALA residues in cereals grain, rape (grains and oil), and fiber flax (oil).

Up to 70% of the plants protector residues can enter a human body with food

products; these residues occur in all media. In this case calculated safe HE-ALA introduction with food products amounts to 3.85 mg/human/daily.

When determining maximum permissible HE-ALA residues level in cereals grain (0.1 mg/kg) and rape (0.6 mg/kg), flax and rape oil (0.6 mg/kg) we allowed for average daily standards of food products consumption.

Consequently, probable daily HE-ALA introduction into a human body allowing for all the standards set for various media amounts to 0.11 mg (2 % of PDI) with food products; 0.3 mg (5.5 % of PDI), with water; 0.2 mg (3.6 % of PDI), with air; totally 11.1 % of permissible daily introduction (PDI).

So, as per toxicological assessment results, we managed to give grounds for a number of technical regulations for HE-ALA content in the environment: tentatively safe HE-ALA content in working area air is equal to 0.8 mg/m<sup>3</sup>; in the atmosphere, 0.01 mg/m<sup>3</sup>; tentatively permissible concentration in water reservoirs is 0.1 mg/dm<sup>3</sup>; in soil, 0.3 mg/kg; permissible daily dose is 0.1 mg/kg; maximum permissible concentration in cereals grain is 0.1 mg/kg; in rape (grain and oil), and fiber flax (oil), 0.6 mg/kg.

**Conclusion.** We performed complex toxicological and hygienic examination of HE-ALA, a new plants growth regulator under various regimes, doses and ways of introduction into experimental animals bodies. The examination results allowed us to detect parameters of its toxicity and biological impacts peculiarities which became apparent through skin-resorptive properties, cumulative ones, and properties causing mucous tunics irritation, moderate reproductive toxicity without any substantial signs of gonad-trophic, mutagenic and allergenic impacts on a body. We determined that HE-ALA toxic effects on a body were membrane-trophic and cytotoxic. We determined limiting parameters of HE-ALA hazardous effects in a chronic experiment; basing on these parameters, we calculated a permissible daily dose for a human and created a number of regulations for the substance content in environmental objects (working area air, the atmosphere, water, and soil), food raw materials, and food products (cereals grain, rape grain, rape and flax oil).

The obtained data can be used as initial ones in setting reference safety levels as per allowable health risk criteria.

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*Vlasenko E.K. Peculiarities of toxic effects exerted by hexyl ether of 5-aminolevulinic acid and grounds for working our regulations of its safe production and application. Health Risk Analysis, 2017, no. 3, pp. 118–126. DOI: 10.21668/health.risk/2017.3.14.eng*

Received: 22.12.2016

Accepted: 20.02.2017

Published: 30.06.2017