BIOCHEMISTRY AND BIOTECHNOLOGY

БИОХИМИЯ И БИОТЕХНОЛОГИЯ

ISSN 2686-7575 (Online)

https://doi.org/10.32362/2410-6593-2022-17-6-492-503

UDC 615.014.24:615.015.4

RESEARCH ARTICLE

Investigation of the biological activity of the water-soluble C_{60}/poly-*N*-vinylpyrrolidone complex

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Abstract

Objectives. *The study aimed to investigate the biological activity of the C_{60}/poly-N-vinylpyrrolidone (C_{60}/PVP) complex representing a water-soluble fullerene derivative. In vitro and in vivo techniques were used to analyze the effect of the C_{60}/PVP complex on the activity of lactate dehydrogenase (LDH) and evaluate changes in the biochemical parameters of blood serum when per os administered to mice.*

Methods. *In order to determine the activity of a commercial LDH preparation and study the kinetics of this process, the standard Warburg photometric method was used. To assess the effect of polyvinylpyrrolidone (PVP) and the C_{60}/PVP complex on some biochemical parameters in vivo, a study was conducted on two-month-old male white mongrel mice weighing 20 ± 3 g. Determination of biochemical parameters of blood serum was carried out using a semi-automatic biochemical analyzer according to standard methods.*

Results. *The effect of the C_{60}/PVP complex on LDH activity was studied along with changes in the biochemical parameters of mouse blood serum characterizing carbohydrate metabolism. As well as increasing the glucose and pyruvic acid content, the C_{60}/PVP complex was found to reduce lactate content and LDH activity in blood serum along with in vitro LDH activity according to the type of mixed inhibition.*

Conclusions. *The C_{60}/PVP complex and PVP were shown to exhibit biological activity in vitro and in vivo. The C_{60}/PVP complex, representing a mixed-type LDH, was shown to inhibit LDH activity, as well as contributing to a decrease in lactate concentration and an increase in the concentration of pyruvic acid and glucose in blood serum when administered per os to mice. The inhibitory effect of PVP on LDH activity was revealed in both in vivo and in vitro investigations. In vivo, PVP contributes to a decrease in the concentration of lactate in the blood. The less pronounced effect of the C_{60}/PVP complex as compared to PVP alone may be due to the fact that C_{60} molecules are "hidden" in cavities formed in PVP molecules.*

Keywords: *fullerene, C_{60}, C_{60}/poly-N-vinylpyrrolidone, C_{60}/PVP complex, LDH activity, biochemical parameters, biological activity*

For citation: Loginova N.Yu., Chesovskikh Yu.S., Borodulin V.B. Investigation of the biological activity of the water-soluble C_{60}/poly-*N*-vinylpyrrolidone complex. *Tonk. Khim. Tekhnol. = Fine Chem. Technol.* 2022;17(6):492–503 (Russ., Eng.). https://doi.org/10.32362/2410-6593-2022-17-6-492-503

НАУЧНАЯ СТАТЬЯ

Исследование биологической активности водорастворимого комплекса C_{60}/поли-*N*-винилпирролидон

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Аннотация

Цели. *Исследовать биологическую активность водорастворимого производного фуллерена – комплекса C_{60}/поли-N-винилпирролидона (C_{60}/ПВП) – в условиях in vitro и in vivo; изучить влияние комплекса C_{60}/ПВП на активность лактатдегидрогеназы (ЛДГ) в условиях in vitro и in vivo; оценить изменения биохимических показателей сыворотки крови при введении мышам C_{60}/ПВП.*

Методы. *Для определения активности коммерческого препарата ЛДГ и исследования кинетики данного процесса был использован фотометрический метод Варбурга с применением стандартной методики. Для оценки влияния поливинилпирролидона (ПВП) и комплекса C_{60}/ПВП на некоторые биохимические показатели in vivo было проведено исследование на двухмесячных самцах белых беспородных мышей весом 20 ± 3 г. Определение биохимических показателей сыворотки крови проводилось с помощью полуавтоматического биохимического анализатора по стандартным методикам.*

Результаты. *Исследовано влияние комплекса C_{60}/ПВП на активность ЛДГ и проведена оценка изменений биохимических показателей сыворотки крови мышей, характеризующих углеводный обмен. Установлено, что комплекс C_{60}/ПВП увеличивает содержание глюкозы и пировиноградной кислоты, снижает содержание лактата и активность ЛДГ в сыворотке крови по сравнению с контролем, а также снижает активность ЛДГ в условиях in vitro по типу смешанного ингибирования.*

Выводы. *Комплексы C_{60}/ПВП и ПВП проявляют биологическую активность в условиях in vitro и in vivo. Установлено, что комплекс C_{60}/ПВП является ингибитором ЛДГ смешанного типа в условиях in vitro, угнетает активность ЛДГ в условиях in vivo, а также способствует снижению концентрации лактата и увеличению концентрации пировиноградной кислоты и глюкозы в сыворотке крови при введении мышам C_{60}/ПВП per os. При этом также выявлено ингибирующее действие и ПВП на активность ЛДГ in vitro и in vitro, причем в условиях in vivo ПВП способствует снижению концентрации лактата в крови. Менее выраженное действие комплекса C_{60}/ПВП по сравнению с ПВП может быть связано с тем, что молекулы C_{60} «скрыты» в полостях, образованных в молекулах ПВП.*

Ключевые слова: *фуллерен, C_{60}, C_{60}/поли-N-винилпирролидон, комплекс C_{60}/ПВП, активность ЛДГ, биохимические показатели, биологическая активность*

Для цитирования: Логинова Н.Ю., Чесовских Ю.С., Бородулин В.Б. Исследование биологической активности водорастворимого комплекса C_{60}/поли-*N*-винилпирролидон. *Тонкие химические технологии*. 2022;17(6):492–503. https://doi.org/10.32362/2410-6593-2022-17-6-492-503

INTRODUCTION

The influence of C_{60} fullerene derivatives on biological objects is being actively studied due to their potential for various preparations in medicine and pharmacology. Due to their widespread application in various industries, as well as currently active research for areas of their future application in the medical and pharmaceutical fields in the diagnosis and treatment of diseases, human interaction with such materials has become an integral part of contemporary life. Since the effects of such materials, which are foreign to the organism, can occur directly or following interaction with biomolecules to include significant metabolic changes, an additionally important task involved in contemporary research is the constant assessment of potential risks when interacting with nanomaterials, including identifying and evaluating their potentially toxic properties.

Recent studies on the properties of a number of C_{60} fullerene derivatives have demonstrated their ability to inhibit HIV-1 protease [1, 2] as a result of their antimicrobial [3] and antioxidant [4] activities. There are also works devoted to the use of fullerenes in the treatment of inflammatory processes [5]. Derivatives of metallofullerenes, in which a metal atom is trapped inside a fullerene cage, are considered especially promising for radiomedicine applications due their greater stability as compared with currently used chelate complexes.

An analysis of the experimental data given in the literature shows that the biological activity of fullerene is generally determined by its lipophilicity (describing its adhesiveness to proteins and lipids along with various membranotropic properties), electron-withdrawing activity (promoting interaction with free radicals and reactive oxygen species), as well as photoexcitation characteristics (ability to transfer energy from an excited state to a molecule of ordinary oxygen and transform it into singlet oxygen). The ability of fullerene to prevent apoptosis of neurons was revealed in a number of works carried out both *in vivo* and *in vitro* [6, 7]. There are data in the literature on beneficial effects obtained using fullerenes and their derivatives in the treatment of neurodegenerative diseases [8].

Fullerene derivatives have been shown to inhibit the chain reaction of lipid peroxidation [6, 7]. The antioxidant properties of fullerenes are largely due to the system of conjugated double bonds capable of accepting additional electrons, for example, from free radicals. One fullerene molecule can accept up to 34 methyl radicals, capture and inactivate superoxide anion radicals and hydroxyl radicals *in vivo* and *in vitro* [7]. In addition, the use of fullerenes as antioxidants

is due to their ability to localize inside mitochondria and other compartments where free radicals are produced. According to the authors of a number of works, fullerenes are effective neuroprotectors in some forms of sclerosis. Due to evidence of improved cognitive processes under normal and pathological conditions, they are of great interest for the development of therapies for Alzheimer's disease [9, 10].

An analysis of the data available in the literature on the study of the biological activity of C_{60} and of its derivatives in *in vitro* and *in vivo* experiments reveals no serious toxic effects from C_{60} fullerene. The first studies on fullerene toxicity showed [11–13] no side effects in mice when observed over long periods following administration of fullerene at doses of 2.5 g/kg. Data on the effects of aqueous dispersion of C_{60} stabilized by starch [14] also show the absence of chronic toxicity when administered intragastrically to rats, as well as causing no significant differences in hematological and biochemical parameters as compared to the control.

Tests of hydroxylated $C_{60}(OH)_n$ fullerene showed that when it was administered intraperitoneally to mice and rats at a median lethal dose LD_{50} of 0.5–2.4 g/kg [15]. At the same time, the authors of the work noted a decrease in the activity of microsomal enzymes—in particular, P_{450}-dependent monooxygenase—in the presence of the hydroxylated form of C_{60} fullerene (*in vivo*).

Fullerene radically differs from all other biogenic molecules due to its spherical structure. Due to inevitable interactions with molecules having hydrophobic regions (as for example in the case of proteins and lipids) in a physiological hydrophilic environment, the carbon surface of such molecules will be practically inaccessible for recognition by the immune system [16–18].

In other studies, while C_{60} fullerene itself was shown not have acute toxicity, toxic effects have been associated with the organic solvents used to prepare fullerene solutions [19]. A more detailed study on the mechanisms of action and the mechanisms of the response of the organism as a whole to the use of both fullerenes itself and its derivatives is hampered by low solubility in the aquatic environment.

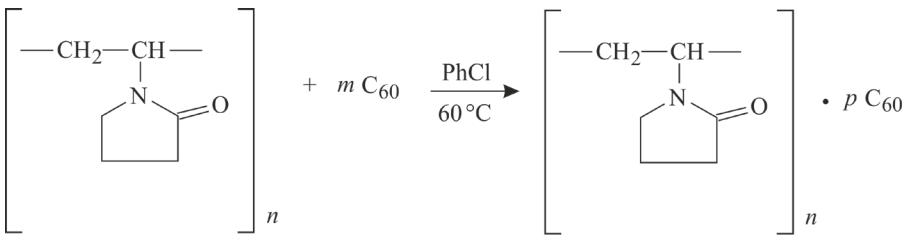
One of the solvents used in medical practice for hydrophobic drugs is polyvinylpyrrolidone (PVP). PVP is non-toxic and included in such medicines as hemodez and enterodez. Medical-grade PVP is obtained by *N*-vinylpyrrolidone-2 polymerization. It is used to prolong the action of some drugs, as well as acting as a binder and stabilizer in the manufacture of tableted forms of drugs, etc.

Previously, biological studies of this complex have already been mentioned in the literature. Fullerenes have been used to protect cells against ultraviolet radiation [20]. Ultraviolet irradiation (320–400 nm) provokes the formation of reactive oxygen species, leading to damage and death of skin cells in humans. A water-soluble C_{60}/poly-*N*-vinylpyrrolidone (C_{60}/PVP) complex was tested as protection against such oxidative stress. In this case, it is the ability of fullerene to penetrate into the deep layers of the human skin epidermis, as well as its resistance to oxidative degradation prevent skin aging without photosensitization and cytotoxicity, that makes it a promising substitute for ascorbic acid.

The potential antiviral effect of C_{60} has been studied in a number of studies. The C_{60}/PVP complex was shown to inhibit the reproduction of influenza A and B RNA-viruses, as well as various DNA-based viruses, in particular, herpes simplex virus (HSV-1) [21, 22]. Here, it was found that morphological changes occur as a result of treating influenza type A virus with the C_{60}/PVP complex. Here, a large number of defective virions and virions with a damaged "brush" were identified along with the presence of lipid membrane disorders. The data suggest that the complex interferes with the assembly process in the viral replication cycle to block the self-tuning of mature viral particles.

Thus, the purpose of this work was to study the biological activity, both under *in vitro* and *in vivo* conditions, of a water-soluble fullerene derivative comprising the C_{60}/PVP complex.

To obtain a water-soluble C_{60}/PVP fullerene derivative, the previously described method of C_{60} fullerene complexation with PVP [23] (Fig. 1) was used.



$m > p$

Fig. 1. Scheme of the complexation reaction of fullerene C_{60} with PVP.

This complex is soluble in water and absorbs at 255 and 330 nm. IR spectrum, υ, cm^{-1}: 578, 844, 894, 933, 1077, 1227, 1266, 1375, 1490, 1630–1687, 1703, 2127, 2395, 2575, 2922, 3329, 3493, 3800, 3943.

PVP has good solubility in water and contains cavities in its structure that are close in size to C_{60} molecules. The cavities are easily filled with fullerene molecules, thus forming a water-soluble complex. The studies were carried out for the C_{60}/PVP complex (the C_{60} content in the complex is ~1.6%). The effect of the C_{60}/PVP complex on the activity of lactate dehydrogenase (LDH) *in vitro* was studied, as well as changes in the biochemical parameters of blood serum when C_{60}/PVP was administered *per os* to mice.

MATERIALS AND METHODS

Determination of LDH activity in model *in vitro* experiments

To determine the activity of a commercial LDH preparation and study the kinetics of this process, the Warburg photometric method was used using a standard technique. We used solutions of phosphate buffer, sodium pyruvate, coenzyme-bound hydrogen nicotinamide adenine dinucleotide ($NAD{\cdot}H + H^+$), LDH (*Sigma-Aldrich*, USA). The change in LDH activity was determined in the presence of a solution of PVP and an aqueous solution of the C_{60}/PVP complex, which were experimental samples and were compared with a control sample that did not contain PVP and C_{60}/PVP. The spectra were recorded on a Specord UV VIS spectrophotometer (*Analytik Jen*a, Germany) in the wavelength range from 220 nm to 400 nm. The maximum absorption of LDH was determined spectrophotometrically: it corresponded to a wavelength of 340 nm. Then, sodium pyruvate (not absorbing at λ = 340 nm), PVP or C_{60}/PVP solution, and LDH were sequentially added to the determination cuvette. Optical absorption was measured at certain time intervals during the course of the reaction. The activity of LDH was determined by the decrease in the content of $NAD{\cdot}H + H^+$ (maximum absorption at a wavelength of 340 nm) in the reaction mixture as a result of the enzymatic reduction of pyruvate to lactate.

The enzyme activity was expressed in nanomoles of the substrate converted into the reaction product in 1 min per 1 mg of the enzyme at 25°C and optimal pH. It was calculated by formula (1):

$$a = (\Delta A \cdot V)/(\varepsilon_{340} \cdot d \cdot V_S) \cdot 10^{-9} \quad (1)$$

where ΔA is the change in the optical absorption of $NAD{\cdot}H + H^+$ for 1 min, rel. un.; V is the final volume in the cell after the addition of the last component, mL; ε_{340} is the coefficient of molar absorption of $NAD{\cdot}H + H^+$ equal to 6220 $M^{-1}{\cdot}cm^{-1}$; d is the thickness of the investigated liquid layer, cm; V_S is the volume of the enzyme solution taken for the study, mL.

The enzymatic reaction rate was determined by formula (2):

$$v = (C_0 - C_{1/2})/(\tau - \tau_0) \quad (2)$$

where v is the rate of the enzymatic reaction, $mol{\cdot}L^{-1}{\cdot}s^{-1}$; C_0 is the concentration of $NAD{\cdot}H + H^+$ before the start of the reaction, mol/L; $C_{1/2}$ is half of the total concentration of $NAD{\cdot}H + H^+$, mol/L; τ is the time during which the enzymatic oxidation of 1/2 of $NAD{\cdot}H + H^+$ concentration occurred (s); τ_0 is the reaction start time, s.

The influence of PVP and C_{60}/PVP was also evaluated by analyzing the change in the Michaelis–Menten constant (K_M). To determine K_M, the reaction rate was calculated at various concentrations of the sodium pyruvate substrate.

Determination of biochemical parameters of blood serum

To evaluate the *in vivo* effect of PVP and the C_{60}/PVP complex on some biochemical parameters, whose concentration depends on LDH activity, a study was conducted on mongrel mice. The experimental part of the work was carried out in accordance with the protocols of the Geneva Convention and the principles of good laboratory practice (National Standard of the Russian Federation, GOST R 53434-2009[1])[2] [24].

The control and experimental groups were formed from two-month-old males weighing 20 ± 3 g. The mice were kept under standard conditions with natural changes in lighting occurring in conformity with the normal vivarium regime. All animals had free access to food and water. The mice of the control group were not exposed to PVP and C_{60}/PVP. The first experimental group received a PVP solution, while the second group received the C_{60}/PVP complex. Each study group of the mice consisted of 15 individuals. The daily dose of

[1] GOST R 53434-2009. National Standard of the Russian Federation. *Principles of good laboratory practice*. Moscow: Standartinform; 2010. 12 p.

[2] *Prakticheskoe rukovodstvo po biologicheskoi bezopasnosti v laboratornykh usloviyakh* (*Practical Guide to Biological Safety in the Laboratory*). Geneva: VOZ; 2007. 188 p. (in Russ.).

administered substances was 0.5 mg/kg. The compounds were administered to the mice as a solution (100 μL) for 5 days using a probe. Following their decapitation at the end of the experiment, the biochemical blood serum parameters of the animals were analyzed.

Determination of the biochemical parameters of blood serum was carried out using a semi-automatic biochemical analyzer Screenmaster (*Hospitex Diagnostics*, Switzerland) equipped with a thermostat, photometer and microprocessor.

The methods are unified [25].

In the work, the following biochemical parameters were determined: the concentration of glucose, lactate and pyruvic acid (PVA), the activity of LDH.

Determination of glucose concentration was carried out by the glucose oxidase method. The concentration was measured photometrically at a wavelength of 500 nm (480–520 nm).

The determination of the concentration of lactate in the blood serum was based on the conversion of lactate into PVA and hydrogen peroxide by lactate oxidase; the measurement was carried out photometrically at 546 nm (500–550 nm).

The determination of PVA concentration in blood serum was based on the Umbright method. The concentration was determined photometrically at a wavelength of 430 nm.

The determination of LDH activity in blood serum was carried out according to the Warburg method. The activity was measured photometrically at a wavelength of 340 nm.

To determine all the parameters under study, reagent kits from *Diakon DS*, Russia were used.

Statistical processing of results

Statistical data processing was carried out using the Statistica v7.0.61.0 software package (*StatSoft*, USA). The reliability of difference in the considered indicators of the control and experimental groups was judged by the value of Student's criterion t and the significance level p. At $p \leq 0.05$, the difference was considered statistically significant.

RESULTS AND DISCUSSION

The effect of the C_{60}/PVP complex on the course of carbohydrate metabolism in mice was assessed on the basis of data obtained in the course of biochemical analysis of blood serum.

The main indicator of carbohydrate metabolism is glucose, which is present in most organs and tissues. The concentration of glucose in the blood is the result of glycogenolysis, gluconeogenesis and glycolysis processes. Since it is the main—and for some tissues, the only—source of energy in the cell, maintaining a constant concentration of glucose in the blood is a necessary condition for the normal functioning of the body. Glucose in the body undergoes oxidation with the PVA formation. The latter, depending on the conditions, is oxidized to acetyl-CoA, which enters into the reactions of the Krebs cycle (aerobic conditions), or is reduced to lactate (anaerobic conditions). In turn, as a product of anaerobic glucose metabolism, lactate enters the blood from skeletal muscles, brain and erythrocytes.

The interconversion of PVA to lactate occurs with the participation of the LDH enzyme. LDH is found in all animal and human tissues, especially cardiac and skeletal muscles, erythrocytes, liver and kidneys. Under physiological conditions, the equilibrium of the reaction catalyzed by LDH is shifted towards the formation of lactate. The activity of this enzyme in the blood serum and the relative content of its isoenzymes can be an important biochemical diagnostic test for a number of diseases. LDH activity in the blood serum is a sensitive indicator of hepatocellular damage. A change in activity is usually observed in hepatitis, drug intoxication, cirrhosis, tumors and traumas.

In the course of the studies, the C_{60}/PVP complex was found to double the content of glucose in the blood compared to the control (Table 1). By comparison, PVP does not have a significant effect on changes in blood glucose levels. A reliable decrease in the content of lactate in the blood serum compared to the control group was 55% in the case of the C_{60}/PVP complex and 30% in the case of PVP.

The concentration of PVA in the blood of mice under the influence of PVP is within the normal range, while the introduction of the C_{60}/PVP complex increases it by 27%.

The decrease in lactate content is associated with a decrease in LDH activity under the action of the C_{60}/PVP complex by 7% and PVP by 27% in comparison with the control.

A possible reason for the increase in the level of glucose in the blood under the action of the C_{60}/PVP complex may be a disfunction of glucose entry into the cell. This leads to the activation of compensatory mechanisms, in particular, to the activation of gluconeogenesis, in which lactate can serve as a source for the synthesis of glucose in the cell (as a result, its concentration decreases in the blood). In the process, lactate is converted into PVA (the main a source for the synthesis of glucose) under the action of the LDH enzyme. The content of glucose increased in the blood of experimental animals.

Table 1. Effect of PVP and C_{60}/PVP on the biochemical parameters of blood serum

Biochemical indicator	Control	PVP		C_{60}/PVP		
	$M \pm m$	$M \pm m$	p	$M \pm m$	p	p'
Glucose, mmol/L	7.04 ± 1.08	6.82 ± 0.51	>0.05	14.00 ± 0.94	<0.01	<0.001
Lactate, mmol/L	25.30 ± 0.39	17.64 ± 0.46	<0.001	11.32 ± 0.85	<0.001	<0.001
PVA, mmol/L	0.11 ± 0.01	0.11 ± 0.01	>0.05	0.15 ± 0.01	<0.01	<0.01
LDH, IU	2025.25 ± 23.28	1470.00 ± 20.94	<0.001	1892.00 ± 15.60	<0.05	<0.001

Note: p is the level of probability of differences in comparison with control; p' is level of probability of differences in comparison with PVP. M is the average value of molar concentration; m is the confidence interval of molar concentration.

The high level of significance of the differences between C_{60}/PVP and PVP ($p < 0.01$) suggests that the effect exerted on metabolic processes in mice in the presence of C_{60}/PVP is due to C_{60} fullerene.

The effect of PVP on carbohydrate metabolism in mice results in a decrease in lactate levels and LDH activity. The decrease in lactate levels is probably due to a decrease in LDH activity. This is consistent with the data obtained in studies on the effect of PVP and the C_{60}/PVP complex on LDH activity in an *in vitro* experiment.

It was found that the studied compounds are inhibitors of LDH activity. The reaction rate in their presence decreases, and the Michaelis–Menten constant, on the contrary, increases (Table 2 and Fig. 2).

The conducted studies on the effect of the C_{60}/PVP complex on LDH activity in model experiments carried out in vitro led to the conclusion that the C_{60}/PVP complex has an inhibitory effect on LDH activity. In the presence of the C_{60}/PVP complex, there is a decrease in LDH activity by 57.3% compared to the control, which leads to a decrease in the catalyzed reaction. In the presence of PVP in the system under study, LDH activity was reduced by 70.9% as compared to the control.

Lineweaver–Burk (Fig. 4) and Eadie–Hofstee (Fig. 5) plots were constructed to identify the type of inhibition in the presence of the studied compounds.

A decrease in the maximum rate of the reaction catalyzed by LDH in the presence of PVP and C_{60}/PVP combined with an increase in the Michaelis–Menten constant may indicate a reversible non-competitive inhibition of LDH.

Based on the Lineweaver–Burk and Eadie–Hofstee plots, the type of inhibition in the presence of PVP and the C_{60}/PVP complex was determined as a mixed type of inhibition, in which a decrease

Table 2. Influence of the studied compounds on the LDH activity

Compound	Concentration × 10^{-2}, mg/mL	$K_M \times 10^{-2}$, M[S]	$r_{max} \times 10^{-7}$, mol/L·s	Activity × 10^{-11}, mol/min	Activity, %
Control	–	0.15 ± 0.01	1.34 ± 0.11	0.96 ± 0.10	100
PVP	2	0.32 ± 0.05	0.90 ± 0.04	0.28 ± 0.04	29.1
C_{60}/PVP	2	0.25 ± 0.04	1.14 ± 0.10	0.41 ± 0.01	42.7

Note: K_M is the Michaelis–Menten constant; v_{max} is the maximum reaction rate; M[S] is the substrate molar mass.

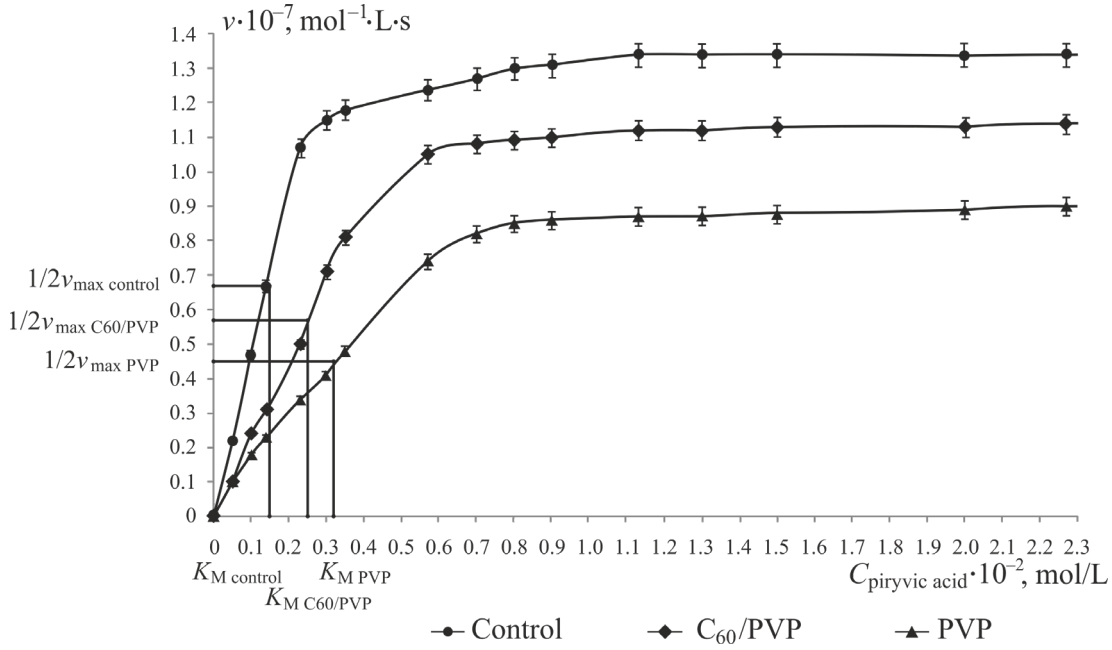


Fig. 2. Graph of the reaction rate (v) versus the substrate concentration (C) in the presence of the compounds under study.

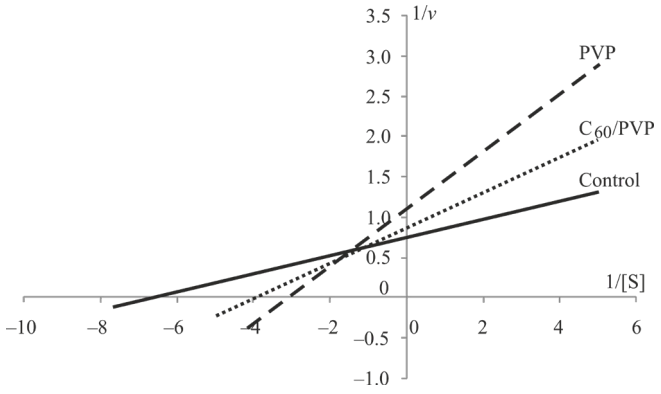


Fig. 3. Lineweaver–Burk graph. S is substrate.

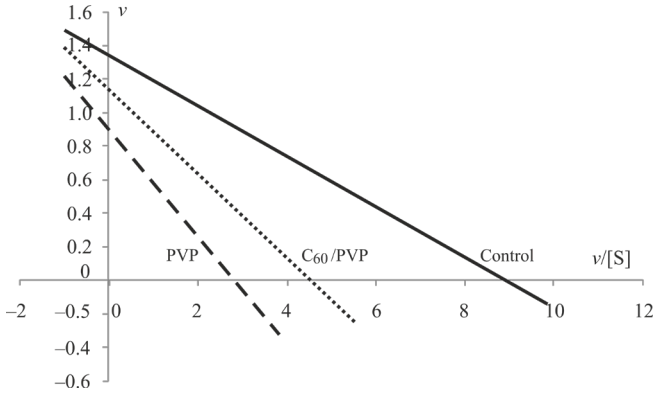


Fig. 4. Eadie–Hofstee graph.

in v_{max} is combined with a simultaneous increase in K_M values. This means that the enzyme–inhibitor complex retains partial activity, i.e., the ability to form an intermediate triple enzyme–inhibitor–substrate complex, in which the substrate undergoes a delayed catalytic transformation.

CONCLUSIONS

The C_{60}/PVP complex and PVP exhibit biological activity under *in vitro* and *in vivo* conditions. It has been established that the C_{60}/PVP complex acts as a mixed-type LDH inhibitor under *in vitro* conditions; when C_{60}/PVP is administered to mice *per os* under *in vivo* conditions, it inhibits LDH activity, as well as contributing to a decrease in lactate concentration along with an increase in the concentration of PVA and glucose in the blood serum. An inhibitory effect of PVP on LDH activity *in vitro* was also revealed; moreover, under *in vivo* conditions, PVP was shown to help to reducethe lactate concentration in the blood. The less pronounced effect of the C_{60}/PVP complex compared to PVP may be due to the fact that the C_{60} molecules are "hidden" in the cavities formed in the PVP molecules.

Authors' contributions

N.Yu. Loginova – collecting and processing materials on fullerene derivatives, a water-soluble C_{60}/PVP complex synthesis, and the study of LDH activity under *in vitro* conditions;

Yu.S. Chesovskikh – collecting and processing materials on fullerene derivatives, the biological activity of the C_{60}/PVP complex *in vivo* study;

V.B. Borodulin – scientific consulting at all stages of the work.

The authors of the article guarantee the absence of a conflict of interest.

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The article was submitted: April 04, 2022; approved after reviewing: May 04, 2022; accepted for publication: November 30, 2022.

Translated from Russian into English by M. Povorin
Edited for English language and spelling by Thomas Beavitt