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Research Article

Determination of the toxicity of zinc sulphate hydrate solution using the Spirotox biotesting method

Elizaveta Evgenievna Kuzmenko*, Quynh Thi Ngoc Hoang, Alena Mikhailovna Koldina, Gleb Vladimirovich Petrov, Anton Vladimirovich Syroeshkin

Department of Pharmaceutical and Toxicological Chemistry, Peoples' Friendship University of Russia (RUDN University), 6 Miklukho-Maklaya St, Moscow, 117198, Russian Federation

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*Address for Correspondence:

Elizaveta E. Kuzmenko, Department of Pharmaceutical and Toxicological Chemistry, Peoples' Friendship University of Russia (RUDN University), 6 Miklukho-Maklaya St, Moscow, 117198, Russian Federation

Abstract

Objective: The objective of this study is to determine the toxicity of zinc sulphate hydrate solutions of different forms using the *Spirostomum ambiguum*.

Methods: An experiment was conducted using a solution of the pharmaceutical substances of $ZnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$. The process of filtration was conducted using a submicron filter. The biological activity of the active pharmaceutical ingredient (API) samples was investigated using the Spirotox method, with the calculation of the activation energy ^{obs}Ea before and after filtration. An energy dispersive X-ray fluorescence spectrometer was used. The dispersed fraction was subjected to analysis by dynamic light scattering (DLS).

Results: The solution of zinc sulphate monohydrate exhibited the highest biological activity and the lowest activation energy (^{obs}Ea) of 68±7 kJ/mol. The solutions prepared from zinc sulphate hexahydrate and zinc sulphate heptahydrate displayed ^{obs}Ea values of 113±5 kJ/mol and 119±5 kJ/mol, respectively, which were found to be equivalent in terms of their biological activity.

Conclusion: The findings of the study, conducted using the Spirotox method, revealed that API zinc sulphate, when administered in different forms of hydration, exhibited disparate biological activity despite the identical concentration. The results of the measurements demonstrated that the method of sample preparation of zinc sulphate solutions of equivalent concentration affects the value of the ^{obs}Ea, which in turn causes different biological activity.

Keywords: zinc sulphate, Spirotox-method, activation energy, bioactivity, toxicity.

INTRODUCTION

Zinc is one of the naturally occurring essential trace elements that is required by living organisms and is of biological importance for the health of the population. Zinc is essential for the normal functioning of over 300 enzymes. It plays an important role in the metabolism of monoamine oxidase, controls DNA synthesis, and it is involved in normal growth, brain development, behavioural responses, bone formation, and other physiological functions [1]. Zinc has indirect antioxidant properties by stabilising cell membrane structures, being a structural component of the antioxidant Cu, Zn-containing superoxide dismutase, and maintaining metallothionein levels [2, 3]. Furthermore, zinc has been demonstrated to exert a direct influence on the immune system, stimulating the proliferation and activation of neutrophils and natural killer cells [4,5].

Zinc deficiency represents the most prevalent cause of secondary immunodeficiency in humans [6]. Zinc deficiency affects approximately two billion people globally and is a significant contributing factor to a multitude of diseases. In children, zinc deficiency results in stunted growth, increased susceptibility to infection and diarrhoea, and is responsible for approximately 800,000 child deaths globally each year. Excessive zinc intake can result in a range of adverse effects, including nausea, dizziness, headaches, abdominal discomfort, vomiting, and even coma. Additionally, it can lead to copper deficiency, altered iron metabolism, and reduced immune function [7-9].

Zinc is less toxic than the majority of other heavy metals. The toxicity of zinc is primarily contingent upon the anion with which it is associated. The mean zinc concentration in adults is approximately 0.5 µmol/g, which equates to a total body zinc content of approximately 2 g [10]. The physiological requirement for zinc in adult males is 1.4 mg/day, while that for females is 1.0 mg/day [11]. The maximum tolerable intake of zinc is set at 40 mg per day [12].

Zinc sulphate ($ZnSO_4$) is a significant pharmaceutical substance containing zinc that is primarily absorbed by the human body in a dissolved state [13]. Zinc sulphate forms a number of hydrates as a result of strong hydrogen bonds with water molecules. These include the heptahydrate ($ZnSO_4 \cdot 7H_2O$), hexahydrate ($ZnSO_4 \cdot 6H_2O$) and monohydrate ($ZnSO_4 \cdot H_2O$) [14, 15]. $ZnSO_4 \cdot 7H_2O$ is also referred to as Goslarite. It is a secondary mineral that is found in the oxidation zone and in old sphalerite mines. It is readily dehydrated and forms minerals.

The thermal dehydration process of $ZnSO_4 \cdot 7H_2O$ comprises several stages, which are outlined in detail in reference [16]:

$$ZnSO_4 \cdot 7H_2O \xrightarrow{39°C - 60°C} ZnSO_4 \cdot 6H_2O + H_2O \quad (1)$$

$$ZnSO_4 \cdot 6H_2O \xrightarrow{60°C - 100°C} ZnSO_4 \cdot H_2O + 5H_2O \quad (2)$$

The above two chemical equations demonstrate that the stable hydrate in equilibrium with water within the range of room temperature to 39°C is $ZnSO_4 \cdot 7H_2O$. $ZnSO_4 \cdot 6H_2O$ exists within the range of 39-60°C, while $ZnSO_4 \cdot H_2O$ exists within the range of 60-100°C. Additionally, the water molecules within the hydrate are also coordinated with the zinc ions. The arrangement of water molecules around the zinc ions is a factor that affects the physical and chemical properties of the zinc ions, leading to significant differences in intermolecular interactions in different hydrates. The three hydrated forms of zinc exhibit distinct crystal structures. The crystal structures of $ZnSO_4 \cdot H_2O$ and $ZnSO_4 \cdot 6H_2O$ are monoclinic, exhibiting distinct lattice parameters. $ZnSO_4 \cdot 7H_2O$, on the other hand, is rhombic in its crystal structure [16]. The relationship between the structure and activity of substances has also been the subject of recent research. This relationship has been demonstrated in numerous studies [17]. Zinc sulphate is employed in the medical field as a food additive [18]. It is employed in the treatment and prophylaxis of zinc deficiency. Despite zinc being an essential element, it is also toxic. High concentrations of free zinc ions in solution have been demonstrated to be toxic to both invertebrates and vertebrates, including fish.

Protozoa play a pivotal role in the functioning of aquatic ecosystems, facilitating the decomposition of organic matter and the conversion of energy to higher trophic levels [19, 20]. From the perspective of microbial ecotoxicology, infusoria lack a cell wall but possess a thin membrane that enables them to absorb chemicals, resulting in alterations to membrane structure. Consequently, infusoria may exhibit altered responses when exposed to metals [21].

Spirostomum ambiguum is an active component of water and wastewater filtration systems. It is 2-3 mm in size and can be observed without the use of a microscope. *S. ambiguum* exhibits considerable resilience to a range of environmental factors, particularly fluctuations in pH levels and dissolved oxygen. Consequently, protozoa are frequently employed as biological indicators of chemical pollution, particularly in aquatic ecosystems [22, 23]. The aim of the study is to evaluate the biological activity of zinc sulphate API solutions with different degrees of wetting at the sample preparation stage using the Spirotox method, in order to see the relationship between the structure and activity of APIs.

MATERIALS AND METHODS

Substances and solutions

In this study, we employed a range of pharmaceutical substances, including $ZnSO_4 \cdot H_2O$ (produced by Acros Organics, Barcelona, Spain), $ZnSO_4 \cdot 6H_2O$ (produced by Fluka, Steinheim, Germany), and $ZnSO_4 \cdot 7H_2O$ (produced by Sigma-Aldrich, USA). All solutions were prepared at a concentration of 0.01M and 0.001M. The prepared solutions were filtered using a submicron filter with a pore size of 0.22 μm (Merck Millipore, USA). The pharmaceutical substance sodium chloride (NaCl, manufactured by Sigma-Aldrich, USA) was dissolved in water to a solution concentration of 0.01M.

The pH of the solution was calibrated through the utilisation of a solution of dilute sulphuric acid (H_2SO_4) and potassium hydroxide (KOH).

The deionised water was purified in a Milli-Q unit at 25 °C (Merck Millipore, USA) to a [D/H] ratio of 140 ppm.

pH measurement

In order to ascertain the pH value of the solution, a basic laboratory pH meter (PB-11, Sartorius, Germany) is utilised.

Spirotox test

A study was conducted to investigate the biological activity of zinc sulphate under different irrigation regimes. The cell biosensor *Spirostomum ambigua*, which is highly sensitive to heavy metals, was employed in this study. The temperature was maintained at a constant level using a 5-well thermoregulated plate, which enabled the temperature to be controlled from 22°C to 28°C in 2°C increments (Lauda Alpha 6, Germany). The behaviour of the infusoria was observed using a binocular microscope, and low-power lamps (10 W) were used.

The introduction of a *S. ambiguum* infusoria into the thermostatically controlled cell of the analysed sample, followed by each of the wells, resulted in the occurrence of cell death at varying time intervals. The period of cellular viability was determined by measuring the time elapsed between the commencement of incubation and the occurrence of cell death. The determination of cell death was conducted through the disruption of cell wall integrity, which resulted in the release of cell contents, or through the immobilisation of cells that exhibited an absence of a contractile response to mechanical stimulation. This methodology is founded upon the principles of the dose-response diagram, as outlined in reference [24]. The activation energy is calculated using the Arrhenius thermodynamic equation [25].

X-ray Fluorescence (XRF)

X-ray fluorescence spectroscopy (energy dispersive X-ray fluorescence spectrometer EDX-7000 (Shimadzu, Japan)) was employed to evaluate the concentration of

impurities present in the native pharmaceutical substances under investigation. The range of elements analysed spanned from 11Na to 92U. The X-ray generator comprised a tube with a Rh-anode, with a current of 1-1000 μA. The method employed was based on the irradiation of the sample under study with X-rays, which resulted in the excitation of the atom. This caused electrons to move to a higher energy level, and upon the reverse transition, the emission of light quanta (fluorescence) was observed. The energy of the radiation emitted (in keV) is a characteristic value for each element.

Dynamic Light Scattering (DLS)

In order to ascertain the dimensions of the nanoparticles present in the aqueous medium, a series of Dynamic Light Scattering (DLS) experiments were conducted on a Zetasizer Nano ZSP instrument, Malvern, UK. The instrument is capable of measuring particle sizes ranging from 0.1 to 1000 nm. Upon irradiation with a laser beam, the particles within the sample scatter the incident light. The movement of particles in a liquid is characterised by a high degree of chaotic scattering in all directions. The particles are in a state of constant motion and collision within the solution, according to the principles of Brownian motion. This results in fluctuations in the intensity of the light scattered.

Statistics

The results of the analyses are presented as mean ± standard deviation (n=5). All calculations and statistical processing were conducted using OriginPro 9.1 software (OriginLab, USA).

RESULTS

Determination of the purity and the absence of impurities

The results of the X-ray fluorescence analysis demonstrated that the APIs were chemically pure, as no other measurable impurities were present. The presence of zinc in the samples tested was demonstrated by the presence of a characteristic peak in the XRF spectra at the Kα and Kβ lines of zinc (8.63 and 9.57 keV, respectively).

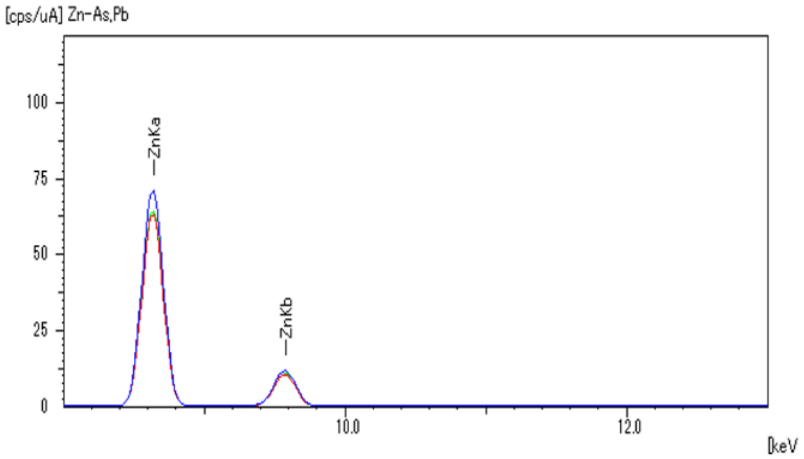

Figure 1: XRF spectrum at characteristic fluorescence energy of zinc (Kα=8.63 and Kβ=9.57 keV)

Determination of the Biological Activity of Solutions $ZnSO_4 \cdot H_2O$ with different concentrations by Spirotox test

The interaction of *S. ambiguum* with the studied solution was found to include the rapid formation of an intermediate state, which then underwent a slow transition to a stationary state. It has been demonstrated that the mortality of *S. ambiguum* exhibits an Arrhenius-type temperature dependence. The mortality rate of *Spirostomum ambiguum* exhibits variation at different zinc concentrations. At a concentration of 0.01 M zinc sulphate monohydrate solution, the mortality rate of the infusoria was higher than at a concentration of 0.001 M.

Table 1: The Biological Activity of Solutions $ZnSO_4 \cdot H_2O$ with different concentrations by Spirotox test.

Temperature (K)	295	297	299	301
Concentration (mol/l)	Lifetime (s) of S.ambiuum (n=5, mean±SD)			
0.01 M	185±3	120±8	106±7	84±4
0.001 M	246±4	185±5	147±3	107±2

It was observed that the lifespan of the infusoria exhibited a difference depending on the concentration of the sample under study. The exposure of infusoria to zinc ions results in their death through the transition state, in accordance with the Michaelis-Menten model of enzymatic kinetics [26]. At each equivalent concentration of zinc, the toxicity of zinc sulfate API solutions with varying degrees of dilution was evaluated in terms of its impact on infusoria.

Measurement of pH value of three zinc sulphate solutions of different forms of watering at different concentrations

The pH of prepared solutions of $ZnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 6H_2O$ and $ZnSO_4 \cdot 7H_2O$ was measured under room conditions (n=3). In accordance with the European Pharmacopoeia, the pH value of a $ZnSO_4 \cdot H_2O$ solution is within the range of 4.0 to 5.6 (C=0.28 mol/l), while that of a $ZnSO_4 \cdot 6H_2O$ and $ZnSO \cdot 7H_2O$ solution is within the range of 4.4 to 5.6 (C=0.18 mol/l). It can be observed that at a concentration of 0.01 M, the pH value attains its maximum value. In conjunction with Table 1, solutions at a concentration of 0.01 M were selected for further optimisation studies.

Determination of biological activity of $ZnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 6H_2O$ and $ZnSO_4 \cdot 7H_2O$ solutions before and after filtration using the Spirotox test

Figure 3 illustrates the activation energy value of ligand-induced death of Spirostomum ambiguum in solutions comprising $ZnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 6H_2O$, and $ZnSO_4 \cdot 7H_2O$ substances at a concentration of 0.001 M, both prior to and following filtration.

The data illustrated in the figure demonstrate that prior to filtration, the lowest activation energy is exhibited by the $ZnSO_4 \cdot H_2O$ solution (68 ± 7 kJ/mol, n = 5), whereas the remaining $ZnSO_4 \cdot 6H_2O$ and $ZnSO_4 \cdot 7H_2O$ solutions display equivalent biological activity with corresponding E_a of 113 ± 5 kJ/mol (n = 5) and 119 ± 5 kJ/mol (n = 5), respectively. The results obtained permit a comparison of the biological activity and toxicity of the hydrated forms of zinc sulphate. The lower value of the activation energy and the shorter lifetime of *S. ambigua* (twofold) in the same temperature range indicate that the $ZnSO_4 \cdot H_2O$ solution exhibits a higher biological activity than the other two solutions.

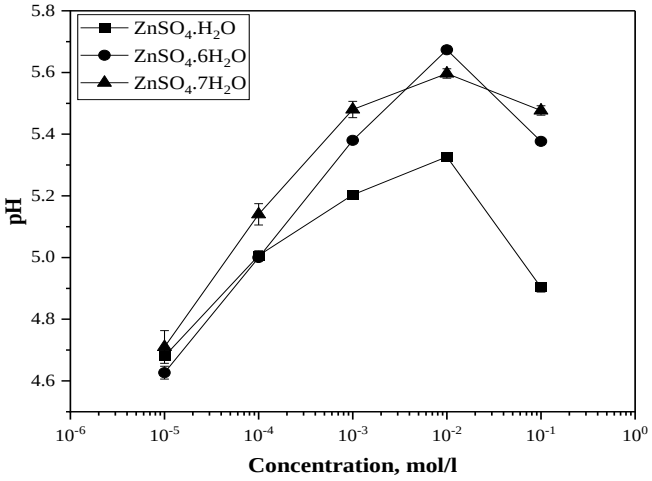

Figure 2: pH value of zinc sulphate solutions of different forms of watering at different concentrations

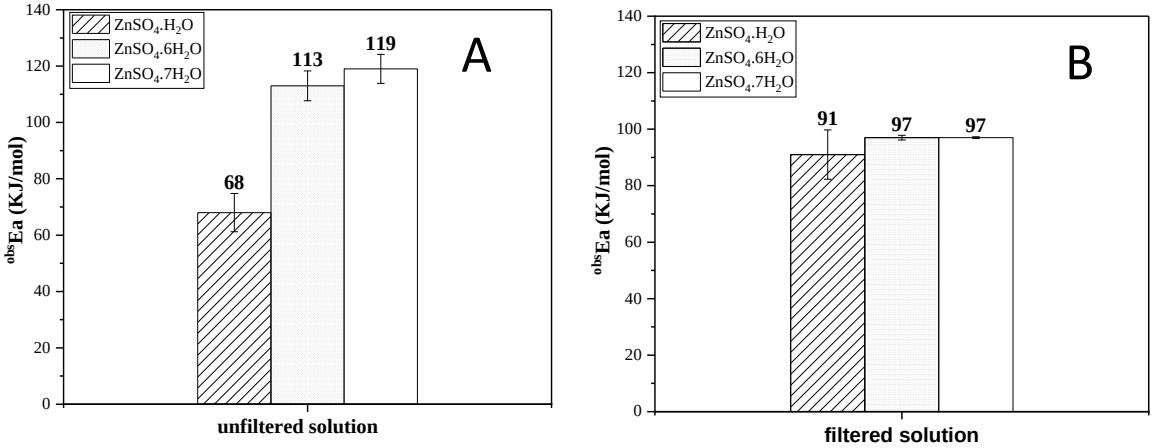

Figure 3: Activation energy of death of *S. Ambiguum* in 0.01M zinc sulphate hydrate solutions as a function of filtration. A – the solutions are prepared immediately prior to the filtration process; B – the solutions were subjected to filtration through a submicron filter.

Figure 3A illustrates the activation energy value of ligand-induced death of Spirostomum ambiguum in unfiltered solutions comprising $ZnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 6H_2O$, and $ZnSO_4 \cdot 7H_2O$ substances. A comparison of the activation energy of the solution prior to and following filtration is presented in Fig. 3a and 3b. It can be observed that the unfiltered monohydrate solution exhibits a distinct activation energy value when compared to the zinc sulfate hexa- and heptahydrate solutions. Following filtration (Fig. 3b), however, all solutions display an identical activation energy value. The discrepancy in the activation energy of the unfiltered solution indicates that the toxicity of the solution is influenced by the size of the clusters and their heterogeneous distribution within the solution. Submicron filtration has been demonstrated to destroy these density inhomogeneities [27].

Determination of cluster size in the studied solutions by Dynamic Light Scattering (DLS) method

Despite comparable concentrations, the three solutions of hydrated zinc sulfate exhibited disparate toxicity profiles. Consequently, we conducted a dynamic light scattering (DLS) analysis to ascertain the dimensions of the nanoparticles in an aqueous solution.

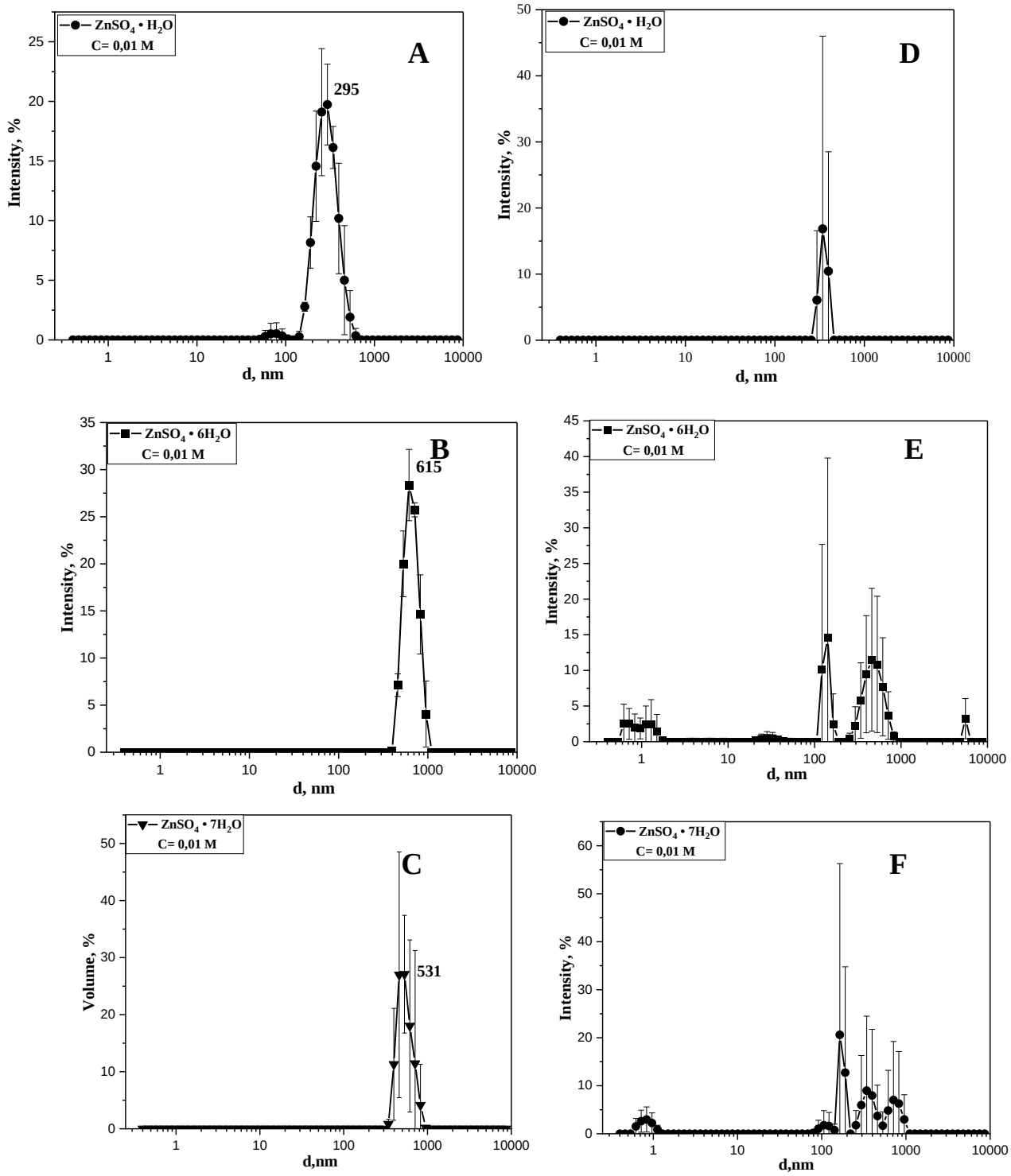

Figure 4: Results obtained by the DLS method, showing the intensity distribution of fractions of the dispersed phase. A, B, C: solutions before the filtering process; D, E, F: solutions filtered through a submicron filter.

Figure 4 illustrates that there is a discernible discrepancy in particle size among zinc sulphate solutions of varying forms of watering. Prior to filtration, the particle sizes of the $ZnSO_4 \cdot H_2O$ solution were observed to be 295 nm, while the $ZnSO_4 \cdot 6H_2O$ solution exhibited a particle size of 615 nm and the $ZnSO_4 \cdot 7H_2O$ solution demonstrated a particle size of 531 nm. Following filtration, no particles were observed within the particle size measurement range of 0.1 to 1000 nm. The formation of hydrogen bonds between water molecules and ions is a consequence of the dissolution of zinc sulphate in water. From this, it can be observed that there is a difference in particle size within the solution.

Determination of the pH value dependence of the solution toxicity

Experiments were conducted on a solution of $ZnSO_4$-H_2O at a concentration of 0.01 M, at two pH levels: 5.2 and 6.5. A control solution of NaCl at the same concentration of 0.01 M was tested at pH values of 5.0, 5.8 and 6.5. As illustrated in Figure 5, an increase in pH from 5.2 to 6.5 was observed to result in a reduction in the lifespan of S. ambigua (from 118 ± 9 s to 67 ± 6 s at 297 K).

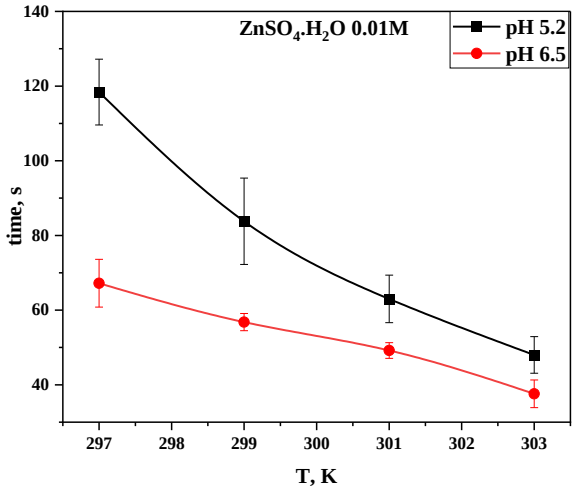

Figure 5: Dependence of *Spirostomum ambiguum* lifetime on temperature at different pH values in $ZnSO_4$-H_2O 0.01M solution (n=5)

The control solution of NaCl 0.01M was observed for 30 minutes, during which no mortality of infusoria was noted (Tab. 2). It is established that S. ambiguum is stable within a pH range of 5.0 to 6.5.

Table 2: Effect of pH value on the lifetime of *S.ambigua* on 0.01M NaCl background solution

NaCl 0.01M			
pH	5.0	5.8	6.5
t, min	> 30 min	> 30 min	> 30 min

The investigation of the toxicity of zinc sulphate monohydrate solution at two different pH levels revealed a correlation between the pH values and the toxicity of the solution. This hypothesis has been corroborated by numerous studies [28, 29]. As illustrated in Figure 4, the toxicity of the zinc sulphate hydrate solution towards the *S. ambiguum* organism exhibited a gradual increase with rising pH levels and approaching neutrality. The influence of the initial pharmaceutical substance's watering on the preparation of solutions was demonstrated.

DISCUSSION

The investigation of the bioactivity of zinc sulphate in three different hydrate forms at a constant concentration revealed that the hexa- and heptahydrate exhibited comparable biological activity. The solution obtained from the pharmaceutical substance $ZnSO_4$-H_2O demonstrated the most pronounced biological activity. In the case of the heptahydrate, six molecules of crystallisation water combine with the zinc ion in a coordination complex. However, the remaining water molecule is weakly bonded to the sulfate ion. Consequently, the water molecule can be readily removed by drying at a constant temperature (at what specific temperature) to form hexahydrate. This may be the reason for the similar energy of the two solutions. Zinc is capable of forming complexes with small molecules, exhibiting coordination numbers 4, 5 and 6: $[Zn(H_2O)_6]^{2+}$, $[Zn(H_2O)_5]H_2O$, $[Zn(H_2O)_4]^{2+}{}_2H_2O$ [30]. It is hypothesised that the number of water molecules in the first and second hydrate shells forms molecular clusters of varying sizes, a conclusion supported by the DLS results.

The results obtained lead us to the assumption that crystals of zinc sulfate hydrate of different watering forms will form different giant clusters with water molecules. The formation of giant heterophase water clusters is facilitated by the formation of aquacomplexes comprising metal cations and various anions, which act as 'centres'. The formation of hydrogen bonds between water molecules and $ZnSO_4$, as well as with zinc ions, results in alterations to the bonding grid space, the molecular arrangement of $ZnSO_4$, and the intermolecular forces. These changes give rise to notable distinctions in the intermolecular interactions observed in the different hydrates. The dimensions of molecular clusters and their distribution within a solution are factors that influence their biological activity. The particle size, as determined by the DLS method, indicates that the particles in the zinc sulphate monohydrate solution are the smallest. This enables them to be evenly distributed and to exhibit the greatest toxicity to infusoria when compared to zinc sulphate hexa- and heptahydrate solutions. The formation of clusters of water molecules, comprising a minimum of six molecules, is a consequence of hydrogen

bonding. A substantial body of literature exists on the formation of water clusters in solution [31]. These clusters are in a constant state of creation and destruction [32, 33]. The size spectrum of these clusters is contingent upon not only the chemical composition but also the history of sample preparation [34]. Submicron filtration represents a means of obviating the necessity for the sample preparation history to be committed to memory, with the consequence that the structures will cease to exist. Following filtration, no particles in the size range of 0.1 to 1000 nm were observed in the solutions under study. It is postulated that clusters may form below 0.1 nm in size or that homogeneous giant clusters may be re-established during the infusoria experiment, resulting in comparable toxicity outcomes.

In water clusters, the interaction between covalent and hydrogen bonds between oxygen atoms and hydrogen atoms enables the migration of protons (H^{+}) by relay mechanism, thereby stabilising the latter. As the size of the clusters increases, their stability also increases up to a certain critical size. Additionally, giant water clusters have been observed to exhibit biological activity [35].

The objective of this study was to further substantiate the correlation between pH and the toxicity of zinc solutions, with a particular focus on drug substances in general. This contributes to the expansion of the research map for infusoria, as there have been numerous studies on the relationship between metal toxicity and environmental pH for vertebrates and microalgae [36, 37], but no such studies have been conducted for infusoria. As illustrated in the Pourbaix diagram of zinc ion properties in water [38], the majority of free zinc ions are present at pH values below 4, with a gradual decline observed from pH 5 to 8. In slightly alkaline conditions, the appearance of $Zn(OH)_2$ and ZnO is observed at pH values between 8 and 10.5. At pH values exceeding 11, the formation of $Zn(OH)_4^{2-}$ ions is noted. Obviously, as the pH is gradually increased to neutral, the amount of free zinc ions gradually decreases. However, for some species of fish and infusoria, an increase in pH will lead to an increase in the toxicity of the solution [39]. Therefore, the toxic impact on infusoria is not attributable to a modification in the acidity index of the medium. Rather, it is a consequence of xenobiotics affecting their cell walls, a factor of considerable significance in the context of Ea and its correlation with LD50 [40], both of which can serve as useful metrics in the initial phase of preclinical toxicity studies of inorganic pharmaceutical substances.

CONCLUSION

The method for determining the toxicity of water-soluble drug solutions on the protozoan *Spirostomum ambiguum* as an alternative to animal testing is an efficient method with ethical value. This method meets the requirements of pharmaceutical research and development under the 3Rs (Refine, Reduce, Replace) strategy, which is oriented towards sustainable ecosystems. The results of the study demonstrate that the zinc sulphate monohydrate solution exhibits the highest toxicity among the three hydrators with observed activity energy (68±7 kJ/mol). The present study demonstrated that the biological activity of the Spirotox test is dependent on the pH of solutions comprising different hydrated forms of zinc sulphate. The Spirotox test can be employed to monitor the toxicity of pharmaceutical substances and to prescribe the less toxic form of zinc sulphate at equivalent dosages in medical practice.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

Contributors: All authors have read and approved the final manuscript.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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