

Article

Influence of D/H Ratio in Water on Fluorescence and Biological Activity of Drug Solutions

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Abstract

The ratio of hydrogen isotopes—protium and deuterium—in water significantly influences the physicochemical and biological properties of dissolved pharmaceutical substances. This study investigated the impact of water isotopic composition on both the kinetic parameters and biological activity of amoxicillin (AMX) and Zn^{2+} solutions. The kinetics of complexation were examined fluorometrically in waters with varying D/H ratios: H_2O (D/H = 145 ppm), heavy water (D_2O , 99.9%), and deuterium-depleted water (DDW, D/H = 10 ppm). The fluorescence intensity of the solutions increased significantly with higher deuterium content in the solvent: $I_{\text{D}_2\text{O}}/I_{\text{DDW}} = 1.5$. The addition of Zn^{2+} induced the changes in fluorescence intensity. The isotope effects $k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}} = 1.9$ and $k_{\text{H}_2\text{O}}/k_{\text{DDW}} = 2.2$ indicate the involvement of water molecules in the rate-limiting step of the process. The decrease in biological activity of AMX due to complexation was confirmed using the biosensor *Spirostomum ambiguum*. Depletion of D/H and $^{18}\text{O}/^{16}\text{O}$ ratios in water increases the biosensor's lifetime. The longest biosensor lifetime was observed in heavy-isotopes-depleted water (HIDW, D/H = 10 ppm, $^{18}\text{O}/^{16}\text{O} = 400$) solutions. Therefore, the variations in the isotopic composition of water from different regions should be considered in the preparation of liquid dosage forms and in the quality control of drugs.

Keywords: deuterium-depleted water; heavy water; heavy-isotopes-depleted water; kinetic isotope effect; amoxicillin; zinc ion; fluorimetry



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1. Introduction

Stable hydrogen isotopes, protium (^1H) and deuterium (D), are components of water molecules. Water with a natural deuterium content is used in pharmaceuticals as a solvent. Deuterium-depleted water is successfully used as an adjuvant in the treatment of oncological diseases [1]. Radiopharmaceuticals “Water (^{15}O) for injection” and “Tritiated (^3H) water for injection” [2] are used in disease diagnostics. Pharmacopoeias from various countries regulate quality requirements for water intended for different purposes (“Water for Injections” [3], “Purified Water” [4]) based on numerous physicochemical and microbiological parameters. To date, the isotopic composition of water—specifically the deuterium/protium (D/H) ratio—has not been considered by pharmacopoeias. Meanwhile, variations in the D/H ratio in natural waters from different regions range from 89.0 ppm, corresponding to Antarctic precipitation (SLAP—Standard Light Antarctic Precipitation), to

155.8 ppm, corresponding to the VSMOW (Vienna Standard Mean Ocean Water) standard for equatorial regions [5]. Waters differ in their physical and chemical properties depending on deuterium content [6,7].

The concentration of the heavy hydrogen isotope deuterium in human blood plasma is 10–12 mmol/L [8]. This level exceeds that of essential macronutrients such as calcium (2.2–2.6 mmol/L) and potassium (3.5–5.0 mmol/L) [9]. Well-known differences exist in the sensitivity of various taxons to changes in the deuterium content of their aqueous environment [10]. The dependence between survival and the observed activation energy ($^{obs}E_a$) of the biosensor *Sp. ambiguum* death and the D/H ratio in the medium [11] resembles trapezoidal “Dose–Response” plots for trace elements [9,12]. The homeostasis zone is limited by both a deficiency and toxic excess of deuterium (Figure 1). The homeostatic zone of deuterium corresponds to its content in natural water. The level of deuterium in human plasma, the shape of the “Dose–Response” curve obtained for the protozoan, and the positive effect of its deficiency on tumor progression in mammals [13] suggest the possibility of considering deuterium as an essential element.

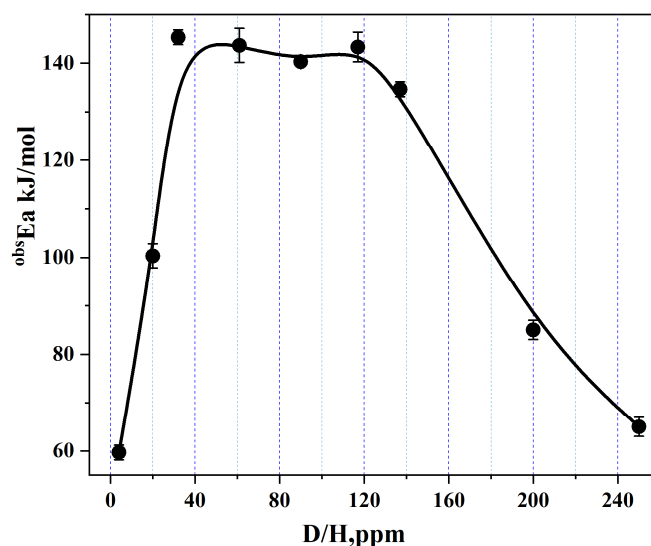


Figure 1. “Dose–response” diagram showing the dependence of the observed activation energy ($^{obs}E_a$) of the biosensor *Sp. ambiguum* death process (mean $\pm$ SD) on deuterium content in water in the range D/H 1–264 ppm [11].

The two-fold difference in atomic mass between the hydrogen isotopes (^1H and D) is the greatest compared to the isotopes of other elements, resulting in a significant kinetic isotope effect (KIE). The D/H ratio in water dramatically alters the physicochemical and biological properties [10] of pharmaceutical substances dissolved in water, as well as the rates of processes involving them [14]. Replacing protium with deuterium in molecules of known drugs, as well as synthesizing new, previously unknown deuterated molecules, has enabled control over the rate of pharmacokinetic processes and the prevention of undesirable biotransformation products [15].

In recent years, there has been growing interest in studying the influence of water isotopic composition—specifically the deuterium-to-protium (D/H) ratio—on chemical processes [16], particularly in complexation involving drug molecules [17]. Although numerous studies have focused on the complexation of amoxicillin with metal ions [18–20], the effect of solvent isotopic composition on the rate and mechanism of this process remains in the initial stage of the research.

Amoxicillin (2S,5R,6R-6-[(2R)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid) is one of the most widely pre-

scribed antibiotics, exhibiting a broad spectrum of therapeutic activity. Its biological efficacy is attributed to the presence of the β -lactam ring, which inhibits the enzymes transpeptidase and carboxylase [21]. The interaction of amoxicillin with metal ions, particularly Zn^{2+} , is of both scientific and practical interest for several reasons. First, zinc plays a crucial biological role as a cofactor for numerous enzymes and is involved in various metabolic processes [22]. Second, the complexation of Zn^{2+} with amoxicillin can significantly alter the antibiotic's physicochemical properties, potentially reducing its antibacterial activity [20].

Fluorimetry is a pharmacopeial analytical method valued for its high sensitivity and specificity. Its wide range of applications includes studying pharmacokinetic profiles and other physicochemical characteristics during drug development and quality control [23]. Fluorescence detection is frequently employed in methods critical to biomedical research, such as flow cytometry [24], fluorescence microscopy [25], fluorescence lifetime imaging microscopy [26], and fluorescence correlation spectroscopy [27].

However, the application of fluorimetry in pharmacopeial quality control is limited due to the dependence of the molecular structure of substances on the physicochemical parameters of the medium, which leads to changes in the spectral characteristics of solutions. The main factors affecting fluorescence are the molecular structure and its prototropic state [28], which vary depending on the pH of the medium [29], as well as solvent polarity, solution temperature [30], and the presence of oxygen, heavy metal ions, and other fluorescence quenchers [31]. The development of fluorometric methods for drug quality control requires careful selection of the solvent, as it must provide high fluorescence intensity and signal stability and avoid quenching. In most quality control methods, the solvent functions not only as a dissolution medium but also participates in chemical and physical processes by interacting with the analyte. Therefore, the choice of a suitable solvent is critically important, particularly in the development of fluorometric methods.

The aim of this study is a deeper understanding of the role of water isotopic composition in both the kinetic parameters and biological activity of amoxicillin and zinc solutions.

2. Materials and Methods

2.1. Chemical Substances

The following substances were used in this work: amoxicillin trihydrate (Ph.Eur. reference standard, EDQM, 99.9%) and ZnCl_2 (Sigma-Aldrich, Saint Louis, MO, USA, 99.9%). For buffer solution preparation, sodium acetate trihydrate (Sigma-Aldrich, Saint Louis, MO, USA, 99.0%), HEPES (Sigma-Aldrich, Saint Louis, MO, USA, 99.5%), and NaOH (Sigma-Aldrich, Saint Louis, MO, USA, 97.0%) were used.

2.2. Water Samples

For solution preparation, deionized high-resistance water ($18.2 \text{ M}\Omega\cdot\text{cm}$ at 25°C ; Milli-Q, Merck Millipore, Darmstadt, Germany) with an isotopic composition of $\text{D}/\text{H} = 145 \text{ ppm}$, $^{18}\text{O}/^{16}\text{O} = 1900 \text{ ppm}$ — H_2O , heavy water with a D_2O content of 99.9% (Cambridge Isotope Laboratories, Andover, MA, USA), deuterium-depleted water (DDW) with a D/H ratio = 10 ppm and $^{18}\text{O}/^{16}\text{O} = 1600$ (Cambridge Isotope Laboratories, Andover, MA, USA) and heavy-isotopes-depleted water HIDW ($\text{D}/\text{H} = 10 \text{ ppm}$, $^{18}\text{O}/^{16}\text{O} = 400$) were used. D/H and $^{18}\text{O}/^{16}\text{O}$ compositions were controlled by multi-pass laser absorption spectroscopy using an Isotopic Water Analyzer T-LWIA-45-EP (Los Gatos Research, Inc., San Jose, CA, USA).

2.3. Buffer Solutions

To prepare 100 mL of a 0.01 M acetate buffer solution with a pH of 5.6, 120 mg of sodium acetate trihydrate was added to 80 mL of water with the required D/H ratio. Then,

0.01 g of glacial acetic acid was added to the solution. The pH of the solution was adjusted to the required final value using HCl or NaOH. The volume of the solution was made up to 100 mL with water.

To prepare 100 mL of a 0.01 M HEPES buffer solution with a pH of 7.0, 240 mg of HEPES was added to 80 mL of water with the required D/H ratio. The pH meter electrode was immersed, and a NaOH solution was added slowly, drop by drop, to raise the pH to the target value of 7.0. The volume of the solution was made up to 100 mL with water.

The pH values of the solutions were measured at 25 °C using a PH400F potentiometer (MT Measurement, Shanghai, China).

2.4. Fluorimetry

Fluorescence spectra were obtained using a Cary Eclipse spectrofluorimeter (Agilent Technologies, Inc., Santa Clara, CA, USA) with two ultrafast scanning monochromators. Measurements were performed with 1 cm closed thick quartz cells at 25 °C. The width of the excitation and emission slit was adjusted to 5 nm. An excitation wavelength of 350 nm was selected by considering the results of the analysis of the test compounds by the method of absorption spectroscopy. The emission spectral range of the measurement was between 350 and 600 nm.

2.5. Spirotox

Individual and combined biological activity of drug solutions was investigated using the test culture *Spirostomum ambiguum*—*Sp. ambiguum* (*Spirostomidae*, *Heterotrichea*, *Ciliophora*).

The laboratory culture was grown in distilled water (D/H = 140 ppm, pH = 6.8–7.2, temperature = 24–26 °C). Under favorable conditions, *Sp. ambigua* cells are characterized by high mobility in both horizontal and vertical directions in the bulk medium, and they do not perish during a period not exceeding the cell cycle (~20 h). Throughout the entire cultivation period, no inhibition of activity or morphological changes were observed in the strain cells. The experimental setup consisted of a 5-well plate with a Lauda Alpha A6 heating thermostat and an MBS-10 binocular microscope. Into each well of the plate, 75 µL of working solutions was added, and 1–2 ciliates were transferred using a pipette. Cell lifetime (t) was calculated as the interval from the moment of incubation to cell death. Cell death was determined by disruption of the cell wall integrity with release of cell contents or by immobilization with absence of contractile response to mechanical stimulation. The mean value was calculated from n = 6 replicates.

2.6. Statistical Data Processing

Experimental data processing was performed using the OriginPro 2021 software package (OriginLab, Northampton, MA, USA). The confidence interval for the mean was calculated using Student's distribution (*t*-test).

3. Results and Discussion

Fluorimetry is a method sensitive to such solvent characteristics as polarity, pH, and temperature. Their control is necessary when studying the kinetics of drug interaction with essential element ions [32]. At the same time, it is important to consider the influence of the isotopic composition of water—the most common solvent used in analytical methods—on the physicochemical and biological properties of pharmaceutical substances dissolved in it, as well as on the rates of processes involving them. In this context, experimental conditions affecting fluorescence intensity—including pH, the isotopic composition of the solvent, and metal ion concentration—were optimized.

3.1. Effect of pH on Fluorescence Intensity of Amoxicillin Solution

According to the pH diagram for amoxicillin, at pH 3.7 aqueous solutions contain zwitterions (II), which are in equilibrium with the fully protonated form (I) (Figure 2). This solution exhibits negligible fluorescence intensity [17]. Solutions with pH values corresponding to the amoxicillin absorption in the human body demonstrated significant differences in fluorescence intensity. Thus, pH 5.6 corresponds to duodenal contents, the site of the onset of amoxicillin absorption [33]. Here, the zwitterionic form (II) predominates, but the fluorescence intensity remains at the same level as at pH 3.7. In contrast, at pH 7.0, corresponding to the pH of the small intestine, the main site of amoxicillin absorption, the solution contains not only the zwitterion but also the fully deprotonated form (III). In this case, the fluorescence intensity increases six-fold.

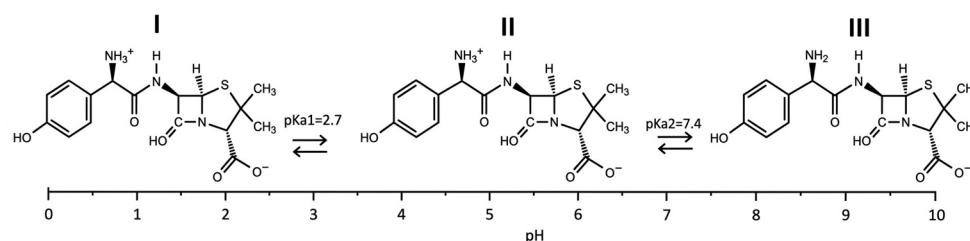


Figure 2. Demonstration of the amoxicillin predominant form in aqueous solutions as pH diagram, adapted from [17].

One factor affecting fluorescence intensity is the length of double-bond conjugation. At pH 7.0, deprotonation leads to the formation of form (III), enabling the lone electron pairs on the amino group nitrogen atoms -NH_2 and -NHR —as well as the oxygen atom of the adjacent carbonyl group—to participate in conjugation with the π -system of the benzene ring. This extension of conjugation length consequently results in an increase in fluorescence intensity. Furthermore, one might hypothesize that the increase in fluorescence intensity arises from excited-state intramolecular proton transfer (ESIPT) [34]. However, the amoxicillin molecule does not meet the necessary criteria [35], as the phenolic hydroxyl group—acting as a photoacid—lacks proton acceptors in the p-position. This is confirmed by the absence of shifts in emission maxima across various pH values [36].

Based on these results, the pH of 7.0 was selected as optimal for all subsequent experiments.

3.2. Influence of the D/H Water Ratio on the Fluorescence Intensity of the Solutions

The D/H ratio of water significantly affects the fluorescence intensity of amoxicillin solutions (Figure 3). These phenomena may be caused by several mechanisms, including energy transfer to the solvent, differences in the spectral properties of water with varying deuterium content, excited-state proton transfer (ESPT), and isotope exchange [37].

The lowest fluorescence intensity was observed in deuterium-depleted water (DDW) with a D/H ratio of 10 ppm. Increasing the deuterium content in the solvent results in a corresponding increase in signal intensity. In H_2O with an isotopic composition, D/H = 145 ppm, the intensity increases by 1.2 times, while in heavy water (D_2O , 99.9% purity), it increases by 1.5 times compared to DDW (D/H = 10 ppm).

According to the literature, the amoxicillin molecule contains four easily exchangeable protium atoms that can undergo hydrogen–deuterium exchange in D_2O [38,39]. Substitution of labile protium atoms in the phenolic hydroxyl (-OH) and amino groups (-NH_2 and -NHR) with deuterium, confirmed by ^1H NMR (Figure S1), may be a key factor in the observed effect. Chemical bonds of deuterium with oxygen and nitrogen (O–D and N–D) exhibit lower vibrational frequencies compared to O–H and N–H bonds, which reduces

the probability of non-radiative vibrational relaxation. This leads to an increase in quantum yield. Furthermore, replacing H₂O with D₂O reduces the efficiency of fluorescence quenching, as energy transfer from the fluorophore to D₂O is less probable [40]. Since the molar absorption coefficients are practically identical for DDW and D₂O, the ratio I_D/I_H can be equated to the ratio of quantum yields (φ_D/φ_H) [37]. Consequently, an increase in the deuterium content in the solvent leads to an increase in the fluorescence quantum yield, with $\varphi_D/\varphi_H = 1.5$.

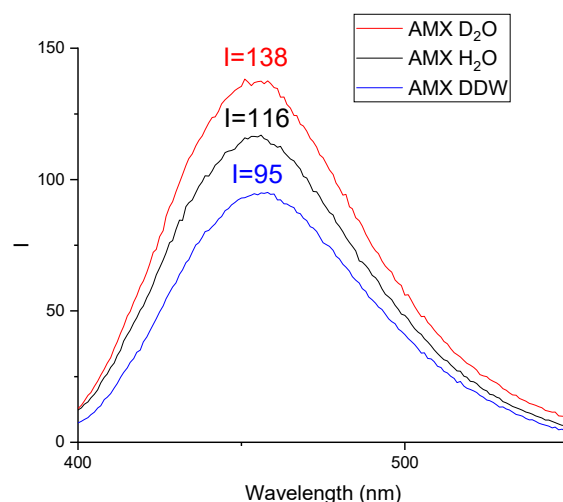


Figure 3. Fluorescence spectra of 4.0 mmol/L amoxicillin solutions at pH = 7.0 in D₂O (99.9%), H₂O (D/H = 145 ppm), and DDW (D/H = 10 ppm), λ_{exc} = 350 nm, T = 25 °C.

The significant increase in fluorescence intensity of amoxicillin solutions in heavy water enhances the sensitivity of the method, thereby improving its effectiveness for drug quality control. Achieving lower limits of detection and quantitation is also crucial for developing fluorimetric methods to determine trace amounts of drugs in ecotoxicological studies [41]. Since amoxicillin is the most widely prescribed antibiotic [42], its accurate detection in trace amounts is crucial for environmental protection. Amoxicillin belongs to a group of antibiotics that undergo virtually no biotransformation in the body. In the environment, it rapidly degrades due to biotic and abiotic factors, resulting in various degradation products [43]. Precise detection of antibiotics at low concentrations is essential for assessing their impact on ecosystems and minimizing the risk of increased antibiotic resistance. In clinical diagnostics and biopharmaceutical research, low limits of detection enable monitoring of antibiotic concentrations in biological samples such as blood or urine, which is vital for optimizing dosage, preventing toxicity, and studying pharmacokinetics [44].

3.3. Effect of Zn²⁺ on Fluorescence Intensity of Amoxicillin Solutions

Metal ions can act as quenchers [45] or enhancers [46] of fluorescence. As demonstrated earlier, cations of s- and d-block elements have different effects on the fluorescence of amoxicillin solutions [17]. Salts of s-block elements, such as Mg²⁺ and Ca²⁺, when added to an aqueous amoxicillin solution, reduce the emission intensity due to the formation of stable non-fluorescent complexes. The effect of zinc content in the AMX solution on fluorescence characteristics was reflected in a hypsochromic shift of the emission maximum by 13 nm (Figure 4). At molar ratios of AMX:Zn²⁺ of 1:0.5 and 1:1, fluorescence intensity increased compared to the solution without zinc. A ten-fold increase in the zinc proportion (1:10) caused fluorescence quenching.

Zn²⁺ ions interact with the amoxicillin molecule through ionic interactions with the carboxyl group and the formation of coordination bonds via a donor–acceptor mechanism. These bonds primarily involve the nitrogen atom of the amino groups. The complexation

of Zn^{2+} ions with amoxicillin molecules at ratios of 1:0.5 and 1:1 results in the formation of structures that are more compact and rigid than the original ones, leading to an enhancement in fluorescence intensity [47]. When the amoxicillin-Zn molar ratio is increased to 1:10, the nature of the effect is reversed. A significant excess of metal relative to the ligand leads to the additional formation of solvent-involved complexes in which an H_2O molecule enters the first coordination sphere as a ligand. The formation of zinc aqua complexes— $[\text{Zn}(\text{H}_2\text{O})_n]^{2+}$ —causes fluorescence quenching due to non-radiative energy transfer to the stretching vibrations of neighboring coordinated O–H oscillators [40]. To confirm the quenching mechanism driven by solvent effects, H_2O was replaced with D_2O .

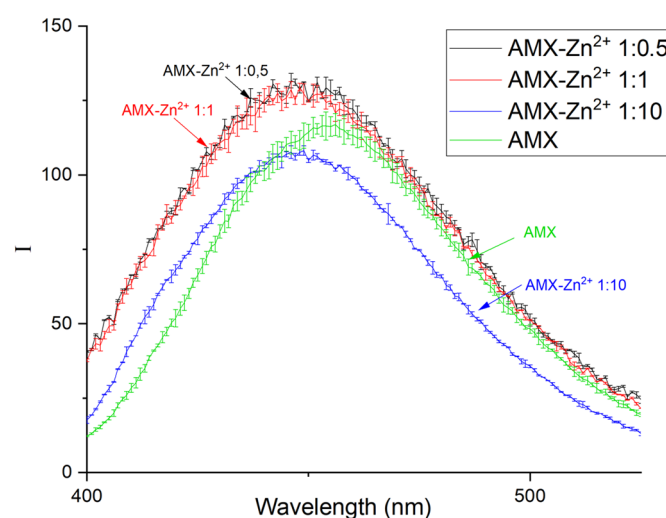


Figure 4. Dependence of fluorescence intensity on the molar ratio AMX: ZnCl_2 in water of natural isotopic composition ($\text{D}/\text{H} = 145$ ppm) at $\text{pH} = 7.0$, $T = 25$ °C.

3.4. Influence of Solvent Isotopic Composition on the Fluorescence Intensity of AMX Coordination Compounds with Zn^{2+}

The isotopic composition of the solvent affects not only the fluorescence of the AMX solution but also that of its complex with zinc ions (Figure 5).

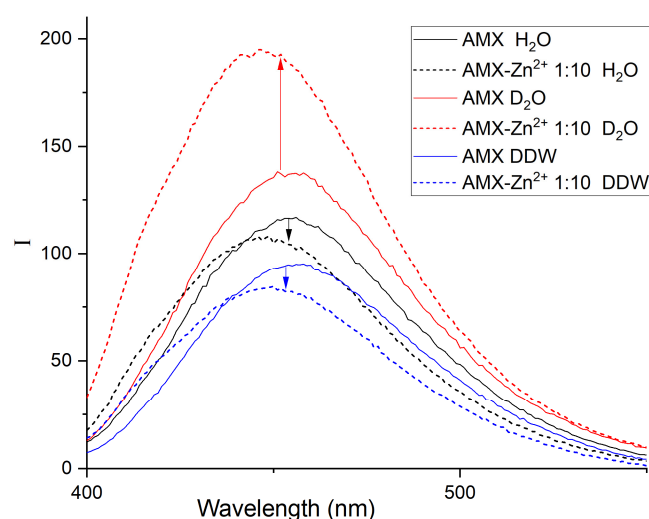


Figure 5. Fluorescence intensity of AMX and its coordination compound with Zn^{2+} (1:10) in waters with different isotopic compositions: H_2O ($\text{D}/\text{H} = 145$ ppm), D_2O (99.9%), and DDW ($\text{D}/\text{H} = 10$ ppm) at $\text{pH} = 7$, $T = 25$ °C.

The introduction of Zn^{2+} ions into an amoxicillin solution caused an immediate hypsochromic shift of the emission maximum to 448 nm in all solvents. This confirms

that coordination compounds of a similar nature are formed in the solutions, regardless of the isotopic composition of the water. Coordination results in fluorescence quenching in H₂O and DDW solutions. In contrast, in heavy water, the addition of Zn²⁺ causes a 1.5-fold increase in signal intensity. In the process under consideration, both H₂O and DDW act as classical quenchers capable of competing with radiative and other non-radiative deactivation processes. As expected, fluorescence is enhanced in D₂O [40].

It was found that at the reagent ratio of 1:10, the fluorescence intensity changes over time, which is associated with conformational changes in the AMX molecule during the formation of the coordination compound.

3.5. Kinetics of Fluorescence in the Complexation of Amoxicillin with Zn²⁺ Ions

As noted above, for the molar ratio of AMX:ZnCl₂ of 1:10, a change in fluorescence intensity was observed. An excess of zinc ions, present as aqua complexes, begins to link monomeric amoxicillin–zinc complexes through the imino group nitrogen atom, the sulfur atom, and the nitrogen atom of the thiazolidine ring. These interactions induce concentration-dependent fluorescence quenching, resulting from the overlap of electron densities and intermolecular π – π stacking [48,49].

This change in fluorescence intensity over time was observed in all the solvents and measured at a wavelength of 448 nm. Previously, for a similar system, we experimentally determined the reaction order using the polarimetric method combined with the Van't Hoff differential equation approach: first order with respect to Zn²⁺ and zero order with respect to amoxicillin [17]. For this fluorimetric research the rate constants for the formation of coordination compounds were determined from the slope of the lines in semi-logarithmic coordinates “Ln(I)-time”. The highest rate of complexation was observed in water with an isotopic ratio D/H = 145 ppm (Table 1). In heavy water, the rate decreased by a factor of two, consistent with results obtained using the polarimetric method [17]. In deuterium-depleted water, the lowest rate of amoxicillin interaction with Zn²⁺ was observed.

Table 1. Rate constants of AMX complexation processes with Zn²⁺ ions at a molar ratio of 1:10 in waters with different isotopic compositions and HEPES buffer (pH = 7), T = 25 °C.

Solvent	$k \cdot 10^4, s^{-1}$
H ₂ O, D/H = 145 ppm, ¹⁸ O/ ¹⁶ O = 1900 ppm	2.26 ± 0.15
D ₂ O, 99.9%	1.22 ± 0.08
DDW, D/H = 10 ppm, ¹⁸ O/ ¹⁶ O = 1600 ppm	1.01 ± 0.02

The calculated isotope effect enables the assessment of the influence of the D/H ratio on interaction rates of AMX and zinc ions. The differences in complexation rates in natural water compared to D₂O and DDW are 1.9 and 2.2, respectively.

To cross-validate the experimental data obtained on the dependence of fluorescence quenching kinetics on the isotopic composition of water, additional polarimetric studies were conducted. The tendency observed in the case of fluorimetric studies was confirmed by the polarimetric experiment (Table S1). The highest values of the interaction rate constant were obtained in water, which is the most heterogeneous in isotopic composition—H₂O.

The kinetic isotope effect we discovered may be related to differences in the symmetry and thermodynamic ordering of the hydration shells of zinc aqua complexes, as well as hydrated amoxicillin–zinc complexes. In solutions prepared with isotopically homogeneous DDW and D₂O, water molecules are rigidly and symmetrically arranged around the metal ion. In isotopically heterogeneous H₂O differences in the lengths and symmetries of the O–H and O–D bonds destabilize the structure of the inner coordination shell. This

locally heterogeneous hydration shell is more easily disrupted, increasing the rate of interaction [50,51].

3.6. Influence of the D/H and $^{18}\text{O}/^{16}\text{O}$ Isotope Ratios in Water on the Biological Activity of Drug Solutions

The observed kinetic isotope effect may reflect changes in the rates of absorption, distribution, biotransformation, elimination, and ligand–receptor interaction mechanisms in the body. It is well known that variations in the deuterium content of water alter its physicochemical properties and significantly impact the dynamics of biochemical, cellular, tissue, and systemic regulatory processes [52]. Producing deuterium-depleted water (DDW) involves reducing not only the heavy hydrogen isotope but also heavy oxygen isotopes, particularly ^{18}O . The content of the ^{18}O isotope in ocean water is 2005 ppm according to the Vienna Standard Mean Ocean Water [6]. Monitoring the content of the ^{18}O isotope is commonly used in studies of various environmental and hydrological processes [53,54]. However, the influence of the $^{18}\text{O}/^{16}\text{O}$ isotope ratio in water on the properties of substances dissolved in it has not yet been addressed in the scientific literature. Therefore, the influence of not only the D/H but also the $^{18}\text{O}/^{16}\text{O}$ isotope ratio of water on the individual and combined biological activity of drug solutions was investigated (Figure 6).

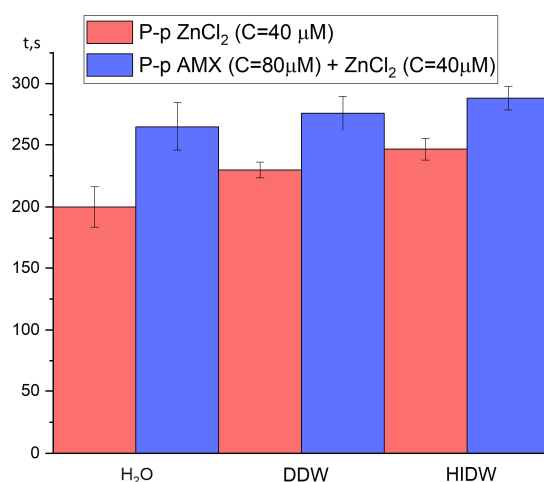


Figure 6. Lifetime of *Sp. ambiguum* biosensor (t) in individual ZnCl_2 solutions (40 μM) and in combination with AMX (80 μM) in waters with different D/H ratios: H_2O (D/H = 145 ppm, $^{18}\text{O}/^{16}\text{O}$ = 1900 ppm), DDW (D/H = 10 ppm, $^{18}\text{O}/^{16}\text{O}$ = 1600 ppm), HIDW (D/H = 10 ppm, $^{18}\text{O}/^{16}\text{O}$ = 400 ppm). Error bars indicate confidence interval ($n = 6$; $p < 0.05$).

It was established that the lifespan of the highly sensitive biosensor *Sp. ambiguum* in individual and combined solutions of the studied substances depends on the isotopic composition of the solvent. As the water was depleted of deuterium and oxygen-18, the biosensor lifetime increased statistically significantly: from 200 ± 12 s in H_2O to 230 ± 5 s in DDW and 246 ± 6 s in heavy-isotopes-depleted water (HIDW). Morphometric characteristics—such as shape, linear dimensions, and mobility of the biosensor—clearly depended on the solvent used. The use of H_2O as a solvent caused a rapid decrease in biosensor mobility and a 2- to 3-fold reduction in size, followed by cell death due to rupture of the cell membrane. In contrast, the use of DDW and HIDW altered the behavior of the ciliate differently. Mobility decreased gradually with periodic contractions; in HIDW, mobility was sustained for a longer period before the ciliate eventually died. The absence of mobility and response to mechanical stimuli indicated the death of the biosensor.

The biosensor lifetime-increasing effect of depleting water of heavy isotopes is also maintained in combined drug solutions. Furthermore, the increased lifespan of

Sp. ambiguum in combined AMX and ZnCl_2 solutions suggests a decrease in biological activity due to complexation. The observed change in the fluorescent properties of the combined amoxicillin– Zn^{2+} solutions in this study also indicates a chemical incompatibility between the pharmaceutical substances.

Thus, the influence of the isotopic composition of water—specifically the content of heavy hydrogen isotopes, such as deuterium (D), and oxygen isotopes, such as ^{18}O —on the biological activity of pharmaceutical substance solutions was demonstrated. The observed isotope effects hold promise for medicine and pharmacy, both in improving drug quality control and in investigating mechanisms of action and methods of xenobiotic detoxification.

4. Conclusions

In this study, the interaction of amoxicillin with Zn^{2+} ions in aqueous solutions of varying pH and water isotopic compositions was investigated using fluorimetry. It was found that the fluorescent properties of amoxicillin are largely determined by its prototropic state: as the acidity of the medium decreases, fluorescence intensity increases due to the involvement of the lone electron pairs on the amino group nitrogen and the carbonyl group oxygen in conjugation with the π -system of the benzene ring. Increasing the deuterium content in water results in an increase in fluorescence intensity. In water with a natural isotopic composition ($\text{D}/\text{H} = 145$ ppm), the intensity increases by 1.2 times, while in heavy water (D_2O , 99.9% purity), it increases by 1.5 times compared to DDW ($\text{D}/\text{H} = 10$ ppm). In heavy water, the enhancement of the fluorescence signal is attributed both to the possible exchange of labile hydrogen atoms with deuterium in the fluorophore and to the reduced probability of energy transfer from the fluorophore to D_2O . The significant increase in fluorescence intensity of amoxicillin solutions in heavy water enhances the sensitivity of the method, thereby improving its effectiveness in drug quality control. This is especially important in the determination of trace amounts of drugs in forensic medicine and ecotoxicological studies.

The addition of Zn^{2+} ions to the antibiotic solution results in a change in fluorescence intensity due to the formation of coordination compounds. The direction of this change depends on the molar ratio and isotopic composition of the solvent. The complexation of amoxicillin molecules with Zn^{2+} ions at ratios of 0.5:1 and 1:1 results in the formation of more compact and rigid structures, leading to an enhancement in fluorescence intensity. A significant excess of the metal to ligand ratio to 1:10 promotes an increase in the proportion of zinc aqua complexes $[\text{Zn}(\text{H}_2\text{O})_n]^{2+}$, which induce fluorescence quenching via non-radiative energy transfer to the stretching vibrations of neighboring coordinated O–H oscillators. Replacing H_2O with D_2O led to a decrease in the probability of proton transfer in the excited state between the chromophore and water, resulting in quenching being replaced by an increase in fluorescence.

A change in fluorescence intensity over time was observed only in solutions with the molar ratio of AMX to ZnCl_2 at 1:10. The calculated isotope effect ($k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}} = 1.9$ and $k_{\text{H}_2\text{O}}/k_{\text{DDW}} = 2.2$) indicates that the isotopic homogeneity of the solvent determines the rate of molecular interactions. The observed differences among H_2O , D_2O , and DDW are attributed to a combination of factors, including the suppression of non-radiative processes. In isotopically heterogeneous water, H_2O , the lengths and symmetries of the O–H and O–D bonds differ, which destabilizes the hydration shells of zinc aqua complexes, as well as hydrated amoxicillin–zinc complexes. This locally heterogeneous hydration shell is more easily disrupted, increasing the rate of interaction in H_2O compared to DDW and D_2O , which leads to fluorescence quenching.

Changes in the content of heavy isotopes in water influence not only kinetic characteristics but also biological processes involving drugs. This study is the first to investigate

the effect of the heavy oxygen isotope ^{18}O content on the individual and combined biological activity of drug solutions. An increase in the survival time of the highly sensitive biosensor *Spirostomum ambiguum* was observed in both individual and combined ZnCl_2 and amoxicillin solutions as the D/H ratio decreased from 145 ppm to 10 ppm and the $^{18}\text{O}/^{16}\text{O}$ ratio decreased from 1900 ppm to 400 ppm in the water. The observed effects associated with the water depletion of heavy isotopes (D and ^{18}O) offer opportunities for medicine and pharmaceuticals in terms of improving the quality control of drugs and xenobiotic detoxification.

Thus, the research demonstrates that fluorimetry is a highly informative method for studying not only complexation processes but also the interactions of molecules with solvents of different isotopic compositions. This study opens prospects for developing new sensitive drug quality control methods with lower limits of detection and quantitation using D_2O as a solvent. The established biosensor lifetime increasing in waters with a reduced content of heavy hydrogen and oxygen isotopes suggests potential applications in xenobiotic detoxification. Furthermore, the differences in the isotopic composition of waters from various regions used in the preparation of liquid dosage forms must be considered according to their quality control using pharmacopeial methods.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/hydrogen7040155/s1>, Figure S1: ^1H NMR spectrum of amoxicillin trihydrate in (a) DMSO- d_6 and (b) D_2O ; Table S1: Rate constants of AMX complexation processes with Zn^{2+} ions at molar ratio 1:10, in waters with different isotopic compositions, acetate buffer (pH = 5.6), $T = 25^\circ\text{C}$.

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Abbreviations

The following abbreviations are used in this manuscript:

AMX	Amoxicillin
DDW	Deuterium-depleted water
HIDW	Heavy-isotopes-depleted water

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