JST&M 10(2026), 30 – 35
DOI: 10.57131/jstm.2026.10.4

Original scientific papers

ELECTROCHEMICAL INFLUENCE OF NIFUROXAZIDE ON DEHYDROGENASE ACTIVITY

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ABSTRACT

Nifuroxazide is an antimicrobial drug from the nitrofuran group, which is used in the treatment of acute bacterial intestinal infections. Its mechanism of action is based on the reduction of the nitro group in bacterial cells, which produces reactive metabolites that permanently damage enzymes and the genetic material of microorganisms. Enzymes of redox metabolism are particularly sensitive, among which lactate dehydrogenase (LDH) stands out. In this work, an electrochemical method (cyclic voltammetry and chronoamperometry) was used to determine kinetic constants. The enzyme LDH (biosensor) was immobilized on a GC electrode, and the effect of nifuroxazide on enzyme activity was monitored. The results show that nifuroxazide binds non-competitively to the enzyme and thus changes the enzyme conformation. This process leads to permanent blockade of key metabolic reactions, disruption of the redox balance, and death of the bacterial cell.

Keywords: biosensors; enzyme; electrochemical method; kinetics;

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1. INTRODUCTION

Nifuroxazide is a nitrofuran derivative, containing a nitro group that can participate in redox reactions. It acts on bacterial enzymes involved in protein synthesis and metabolism, especially those that depend on oxidoreductase processes. The nitro group can be reduced in bacterial cells, creating reactive intermediates that damage enzymes and DNA. This leads to inhibition of bacterial and parasitic growth. Nifuroxazide is a selective inhibitor of bacterial oxidoreductases, and its reactive metabolites secondarily block dehydrogenases, transaminases, and DNA replication enzymes. This achieves a combined effect: blockade of energy metabolism, protein synthesis, and damage to genetic material, which leads to the death of the bacterial cell. The role of dehydrogenases (E.g., Lactate dehydrogenase) is crucial in energy metabolism, and their sensitivity stems from the fact that reactive metabolites of nifuroxazide interfere with electron transfer in these enzymes. The consequence of inhibition is a disruption in the formation of ATP and the energy balance of bacteria. Lactate dehydrogenase inhibitors are

commonly investigated chemical compounds for the treatment of cancer. Most cancer cells switch their metabolism from mitochondrial oxidative phosphorylation to aerobic glycolysis to generate ATP and precursors for the biosynthesis of key macromolecules. Aerobic conversion of pyruvate to lactate, coupled with oxidation of the nicotinamide cofactor, is a primary hallmark of cancer and is catalyzed by lactate dehydrogenase (LDH), the central effector of this pathological reprogrammed metabolism. Therefore, inhibition of LDH is a potential new, promising therapeutic approach for the treatment of this severe, currently incurable disease [1]. The nitro group of nifuroxazide is electrochemically active and undergoes reduction in bacterial cells.

It creates toxic intermediates that interfere with enzymes dependent on oxidoreductive reactions. The mechanism of action of nifuroxazide consists of several steps. The first step is entry into the bacterial cell, where it reaches the intestinal lumen and contacts bacteria, is not absorbed systemically, and acts locally. Then, the nitro group is reduced because bacterial oxidoreductases reduce the nitro group of nifuroxazide, and reactive intermediates (radicals, aldehydes) are formed. The third step in the mechanism is the formation of toxic metabolites that bind to the sulfhydryl groups of enzymes and damage key metabolic enzymes (dehydrogenases, transaminases). Inhibition of protein synthesis and energy metabolism is the fourth step, where the blockade of enzymes leads to disruption of protein synthesis, and energy cycles (ATP production) are interrupted. Then follows DNA damage, reactive metabolites attack DNA, while DNA replication and repair enzymes are inhibited. The final step in the mechanism is the death of the bacterial cell, where a combination of enzyme blockade and DNA damage leads to a bactericidal effect. Enzyme inhibitors currently represent almost half of the drugs in clinical use; therefore, selective inhibition of enzymes of infectious organisms (e.g., viruses, bacteria, and multicellular parasites) is an attractive means of chemotherapeutic intervention for infectious diseases. This strategy is well represented in modern medicine, and a significant part of antiviral, antibiotic, and antiparasitic drugs in clinical use today derive their therapeutic efficacy from the inhibition of selective enzymes [2]. In this work, an electrochemical method was used to study the influence and inhibitory properties of nifuroxazide on the activity of the enzyme lactate dehydrogenase (LDH). Direct electrochemistry of the enzyme can provide a good model for investigating enzymatic activity and electron transfer in biological systems [3]. This work aims to determine the binding mode of nifuroxazide by the electrochemical method, determine the type of inhibition, and the kinetic constants.

2. EXPERIMENTAL

Materials: Lactate dehydrogenase enzyme LDH Assay Kit AB102526 Abcam (Cambridge); KH_2PO_4 and Na_2HPO_4, Fisher Chemical (Wien, Austria); Nafion Sigma-Aldrich (Buchs, Switzerland); Nifuroxazid, Bosnalijek, BiH.

Methods: The instrument used for the measurements was a potentiostat/galvanostat Vertex one, Ivium Technologies, with a classic three-electrode system in which a saturated Ag/AgCl electrode was used as a reference electrode, a Pt electrode as a counter electrode, and GC (glassy carbon) as a working electrode. An amperometric biosensor was formed by immobilizing an enzyme (LDH) trapped in a Nafion layer on the surface of the GC electrode, as described in the literature [4,5]. The electrochemical methods used for the measurements were cyclic voltammetry and chronoamperometry.

Cyclic voltammetry was used to investigate the immobilization of the enzyme film on the electrode surface. All cyclic voltammetry tests were performed in physiological phosphate buffer solution (pH 7) and in the potential range from -1.0 to 1.0

V, at a scan rate of 50 mV/s. Chronoamperometric technique was used to determine kinetic parameters: Michaelis-Menten constant (K_m) and the maximum current value when the solution is saturated with substrate (I_{max}), which is equivalent to the maximum reaction rate (V_{max}), as well as to determine the type of inhibition [6]. Chronoamperometric measurements were performed in a 25 mL phosphate buffer cell at a constant potential of 0.9 V imposed on the working electrode, as well as at constant stirring of 400 rpm. The reaction was observed in the absence and presence of different concentrations of nifuroxazide.

3. RESULTS AND DISCUSSION

In an enzyme sensor, the signals generated by enzymatic reactions are converted into electrical signals by a GC electrode [7,8]. The immobilized enzyme exhibits good sensitivity, selectivity, and response time. Many types of dehydrogenases catalytically transport electrons to their respective electron acceptors. The kinetic parameters obtained by the analysis based on the Michaelis–Menten equation may include the effect of mass transfer resistance through the outer membrane of the enzyme, but the effect is estimated to be small [9]. Detailed mechanistic studies of electron transfer between the electrode and the enzyme can be very complex, especially if the enzymatic reaction is multi-step. In our work, the LDH I substrate reaction proceeds according to a simple mechanism and with high efficiency.

The enzyme LDH catalyzes the reversible conversion between pyruvate and lactate, using the coenzymes NADH/NAD^+:

Pyruvate + NADH → lactate + NAD^+ where:

Pyruvate: main product of glycolysis, substrate of LDH under anaerobic conditions
NADH: reducing equivalent, brings electrons to convert pyruvate to lactate.
Lactate: product of the reaction, important for the regeneration of NAD^+, which allows glycolysis to continue.

This reaction is crucial in anaerobic metabolism in muscles during intense exercise, as it ensures the regeneration of NAD^+ and thus maintains ATP production.

Figure 1 shows cyclic voltammograms of a GC electrode without and with immobilized LDH in phosphate buffer, pH 7, at 50 mV/s. Polymers and other materials are used to immobilize LDH on a GC electrode, as well as other enzymes, and their efficiency depends on the thermodynamics of the redox reaction in the biosensor, the kinetics of electron transfer between the biosensor and the electrode, and the charge transfer within the film on the electrode (biosensor) [10]. Films thicker than a monolayer of electroactive enzyme can often provide a higher enzyme loading per unit electrode area, resulting in higher current peaks.

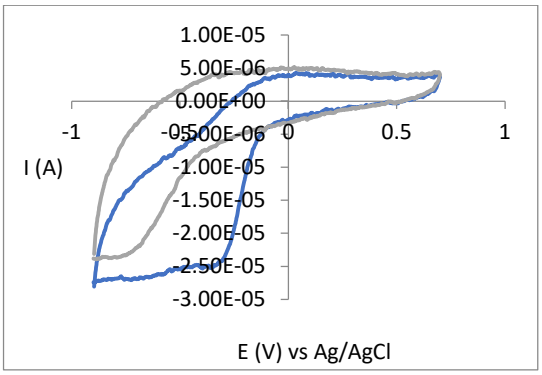


Figure 1. Cyclic voltammogram without enzyme immobilization (gray color) and with immobilized enzyme (blue color)

The catalytic reduction current was monitored by the chronoamperometric method in the presence of different substrate concentrations (1 − 5 mM) in physiological solution. The type of inhibition and the values of the maximum current (I_{max}) and Michaelis-Menten constant (K_m) were determined on the Lineweaver-Burke diagram without and in the presence of the inhibitor, Figure 2.

The Lineweaver-Burk diagram shown in Figure 2 shows that non-competitive inhibition is present in the performed enzyme reactions. In Figure 2, we see that

adding nifuroxazide to the reaction mixture results in the binding of nifuroxazide to the enzyme-substrate complex.

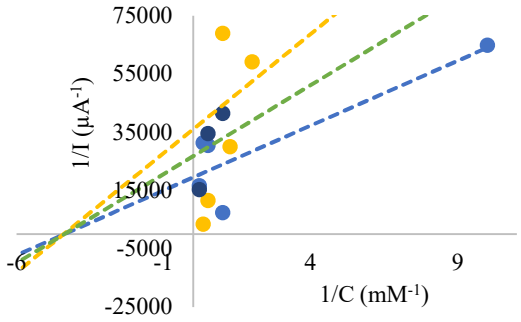
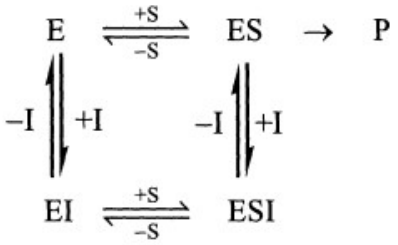


Figure 2. Lineweaver – Burk diagram for determining I_{max} and K_m in the absence and presence of different concentrations of niforoxazide – 0 mM; - 1.025 mM; - 2.023 mM

This type of inhibition is characteristic in that it depends on the presence of the substrate and does not affect the enzyme's affinity for the substrate. In the absence of an inhibitor, the enzyme binds the substrate to form an enzyme-substrate complex, which then catalyzes the reaction and converts the substrate into the product, reflecting the maximum rate at which the enzyme can convert the substrate under optimal conditions. In non-competitive inhibition, the inhibitor specifically binds to the enzyme-substrate complex, changing its conformation. As a result, the enzyme can no longer catalyze the reaction at the same rate as in the absence of inhibition. This reduces Vmax because it reduces the number of functional enzyme sites for catalyzing the reaction. The Michaelis-Menten constant (K_m), which reflects the affinity of the enzyme to the substrate, remains unchanged in noncompetitive inhibition because the enzyme can still bind the substrate with the same affinity, although it cannot catalyze the reaction at the same rate as in the absence of inhibition. A preincubation time of 10 minutes is sufficient to cause a conformational change in the structure of the enzyme and to achieve the binding of nifuroxazide to the ES complex to reduce changes in heart, muscle, or brain cells. Mechanism:



The obtained values of kinetic constants I_{max} and K_m change with the addition of different concentrations of inhibitors, and are shown in Table 1.

Table 1. K_m and I_{max} values in the absence and presence of different concentrations of nifuroxazide

[Nifuroxazide] (mM)	$I_{(max)}$ x10^{-4} (A^{-1})	K_m (mM)
0	5.132	0.22
1.025	3.738	0.22
2.023	2.777	0.22

Nifuroxazide binds to an allosteric site on LDH, altering its catalytic efficiency but not affecting the enzyme's affinity for the substrate. This may have important implications in a metabolic context, as LDH plays a key role in anaerobic glycolysis (the conversion of pyruvate to lactate). Inhibition of LDH could reduce the proliferation of cells that depend on lactate metabolism, which is particularly relevant in investigating the antitumor effects of nifuroxazide. Nifuroxazide is known to be a STAT3 inhibitor, leading to changes in glucose and lipid metabolism in hepatocytes and tumor models [11]. These effects indirectly indicate that the drug may affect glycolytic and fermentative enzymes, including LDH, as LDH is crucial for maintaining anaerobic metabolism in tumor cells. Studies in breast cancer models have shown that nifuroxazide reduces angiogenesis via VEGF signalling [12,13]. The combination of antiangiogenic effects and potential LDH inhibition suggests that the drug may limit the proliferation of lactate-dependent tumor cells. Recent

studies have revealed that NFX has many promising biological activities beyond its classical uses, such as its involvement in anti-inflammatory, antioxidant, and potential roles in inhibiting thyroid, breast, lung, bladder, colon, and liver cancers, as well as osteosarcoma, melanoma, and multiple myeloma. Furthermore, it has promising effects against sepsis-induced organ damage, liver disorders, kidney disease, ulcerative colitis, and immunological disorders. NFX exerts many biological effects, mainly mediated by STAT3 inhibition [14]. Table 2 provides the relationships between the electrochemical effect, biological consequences, and pharmacological implications [15,16].

Table 2. Relationship of the electrochemical effect of nifuroxazide on LDH with biological consequences and pharmacological implications

Electrochemical effect	Biological consequence	Pharmacological implication
Decrease in (I_{max}) (electron transfer capacity)	Reduced NADH oxidation → weaker NAD^+ regeneration	Limits glycolysis in bacteria and tumor cells
Unchanged (Km) (substrate affinity)	Substrate still binds, but enzymatic efficiency decreases	Does not alter binding, only reduces functional enzyme capacity
Non-competitive inhibition of LDH	Disruption of cellular redox balance	Antibacterial effect in intestines; potential antitumor activity
Reduced NAD^+ regeneration	Cells lose the ability to sustain anaerobic metabolism	Tumor cells lose proliferative advantage (Warburg effect)
Blockade of part of the active sites	Fewer available channels for electron transfer	Selective reduction of metabolic activity in pathogenic microorganisms

Electrochemical data clearly show that nifuroxazide acts as a non-competitive inhibitor of LDH. Biologically, this means disruption of NAD^+ regeneration and glycolysis, while pharmacologically, it explains its antibacterial application and potential antitumor perspective. [17,18,19,20].

4. CONCLUSION

Electrochemical data clearly show that nifuroxazide acts as a non-competitive inhibitor of LDH, reducing the electron transport capacity without changing the substrate affinity. Biologically, this means a disruption in the regeneration of NAD^+, which directly affects the metabolism of glycolysis and may have antimicrobial and antitumor significance. This process leads to a permanent blockade of key metabolic reactions, disruption of the redox balance, and death of the bacterial cell.

Acknowledgments

This research was supported by funding through grant number 05-35-3573-1/25 provided by the Federal Ministry of Education and Science, Sarajevo.

Conflicts of Interest

The authors declare no conflict of interest.

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