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ELMANN METHOD FOR ENZYME INHIBITION

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ABSTRACT

Enzyme inhibition plays a crucial role in understanding enzyme kinetics, drug discovery, and biochemical regulation. Determining the inhibitory effects of various compounds on enzymatic activity provides valuable insights into their potential therapeutic or toxicological properties. The Ellman method, based on the colorimetric detection of thiocholine produced by the hydrolysis of acetylthiocholine, is one of the most widely used and reliable spectrophotometric techniques for assessing cholinesterase enzyme activity. In this study, the inhibitory effects of selected compounds on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were evaluated using the Ellman assay. The method involves the reaction of the thiol group of thiocholine with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to produce a yellow-colored 5-thio-2-nitrobenzoate anion, measurable at 412nm. Kinetic analyses were performed to determine inhibition types and constants (Ki values). The obtained data demonstrated that the tested compounds exhibited varying degrees of inhibition, suggesting their potential use as enzyme modulators or drug candidates. The study confirms the efficiency, sensitivity, and reproducibility of the Ellman method for enzyme inhibition analysis, emphasizing its importance in biochemical and pharmacological research.

KEYWORDS: Enzyme Inhibition, Ellman Method, Cholinesterase, Spectrophotometry, Kinetic Analysis.

1. INTRODUCTION

The Ellman method was developed in 1959 to bolster screening capabilities for biologically active substances. Proposed by George Ellman, it has become internationally recognized as the standard assay for acetylcholinesterase enzyme inhibitors. The method has found diverse applications spanning pharmaceutical research, agrochemical development, and toxicology. (Bartolini2024; Li et al.2022). Its popularity stems from several inherent advantages,

including a direct relationship between substrate and product concentrations, the absence of toxic reagents, and high sensitivity, which facilitates monitoring even trace amounts of inhibitor residue. (Dong et al.2024; Chen et al., 2025). The assay is rapid, conclusive, and applicable in environments lacking an analytical laboratory, enabling simple, low-cost derivatization of mercapto compounds. It remains prominently recommended for assaying cholinesterase activity on tissues and in human fluids (Lamba & Pesaresi, 2022). The Ellman method is based on the quantitative determination of sulfhydryl groups using Ellman's reagent (5,5′-dithio-bis-2-nitrobenzoic acid, DTNB). A colorimetric assay for measuring the activity of cholinesterase enzymes also exists and is the method of choice for determining the inhibitory potency of potential antidotes (Ellman, 2022; Cao et al., 2025)(Jain et al., 2024; Lamba & Pesaresi, 2022). The Ellman method relies on the nucleophilic attack of a thiolate anion on the disulfide bond of Ellman's reagent to generate a mixed disulfide and 5-thio-2-nitrobenzoate (TNB^{2-}). In aqueous solution at neutral to slightly alkaline pH, TNB2−, which is not fluorescent, exists and absorbs strongly at 412 nm (ε = 13,600 $M^{-1}cm^{-}1$). This reaction is highly specific and much faster with thiols than with other nucleophiles. Before the introduction of this reagent, the assay of thiols had to deal with tedious and nonspecific methods. (Monga & Nandini, 2024; Younas et al.2023; Yadav et al.2025; Ferrer-Suetaa, 2022). In a typical enzyme activity assay using the Ellman method, the substrate acetylthiocholine is hydrolyzed by the enzyme, producing thiocholine and acetate. Thiocholine, the leaving group, reacts with Ellman's reagent to liberate TNB^{2-}, which absorbs at 412 nm. Increasing absorbance at this wavelength can be used to follow the kinetics of enzyme activity, allowing an estimation of the enzymatic activity. Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), DTNB) reacts with thiol groups to generate 2-nitro-5-thiobenzoate (TNB), which produces a yellow color that is monitored at 412 nm and quantifies the thiol groups in a solution. Enzymes are biological molecules that catalyze specific biochemical reactions either increasing or decreasing the rate of such reactions. Inhibition of an enzyme can be defined as the decrease in the enzymatic activity altering the rate of enzymatic reaction in the presence of other chemicals. The molecules that are responsible for the reduction of the rate of enzymatic reactions are called inhibitors. Enzyme activity or inhibition can be measured by incubating the enzyme (acetylcholinesterase, AChE) with the inhibitor (Jain et al., 2024; Yadav et al.2025; Souza et al.2024; Nakládal et al.).

The Ellman method is a specrophotometric method for the detection of AChE activity and is based on the hydrolysis of acetylthiocholine and the reaction of the released thiocholine with Ellman's reagent (DTNB). Enzyme inhibitors are mainly classified into three groups according to their binding sites with the substrates: (i) competitive (ii) non-competitive and (iii) un-competitive. Depending upon the mechanism of inhibition, the IC_{50} value is calculated. IC_{50} (inhibitor concentration) is defined as the concentration of the inhibitor required to reduce the enzyme activity by 50%. The IC_{50} value provides information about the potency and strength of the inhibition and is inversely related to inhibition. The method is highly sensitive to free thiol, and is effective; ten nanomoles of inhibited enzyme or reagent will give a positive reaction. The parameter X_{inh} can be used to determine type of enzyme inhibition; parallel families of curves indicate uncompetitive inhibition, whereas increasing slopes followed by a broad maximum are characteristic of non-competitive inhibition. Inhibition of cholinesterases by the oximes P2AM and toxogonin: Non-enzymic hydrolysis of acetylthiocholine results from spontaneous hydrolysis and hydrolysis catalyzed by oximes. Rates of hydrolysis are measured following thiocholine concentrations using spectrophotometry, and equilibrium dialysis assesses P2AM binding. Inhibition of cholinesterases is formally evaluated by calculating K_m, K_{ss}, and V constants from enzyme activity data. (Souza et al.2024; Yadav et al.2025; Xiao et al.2024). Oxime inhibition is interpreted via a hypothesis in which allosteric substrate-inhibition sites bind reversible inhibitors, thereby preventing substrate hydrolysis at the catalytic site (Simeon et al., 1981). Kinetic modeling of time-dependent enzyme inhibition by pre-

steady-state analysis of progress curves: Initial velocities are independent of inhibitor concentration when reactions start with the enzyme last, indicating slow inhibition; the rate of EI formation is proportional to galantamine concentration, causing delayed steady-state rates. Preincubating with E–I yields progress curves with upward curvature during the early phase, indicating slow inhibitor dissociation. Initial velocities analyzed with the Michaelis–Menten equation can overestimate K_i or suggest apparent uncompetitive inhibition. Lineweaver–Burk plots suggest competitive inhibition with a K_i of 122.8 ± 20.7 nM in enzyme-initiated reactions; substrate-initiated reactions appear mixed-type with K_{ic} of 18.0 ± 1.5 nM and K_{iu} of 52.7 ± 2.7 nM. Analysis accuracy depends on reaction initiation and detection method, affecting the observed inhibition extent (Attaallah & Amine, 2021; Lamba & Pesaresi, 2022; Alhawday et al.2024; Ghannay et al.2024; Pham et al., 2025); Kumari et al.2024).

Since its introduction, Ellman's reagent has served as the cornerstone of diverse methods used daily in biochemical laboratories worldwide for the quantitative determination of the activity of various enzymes. The need for construction and practical testing of a kinetic model suitable for characterizing reversible enzyme inhibition by progress curve analysis thus remains particularly important and is addressed in the present work. (Varshini et al., 2025; Famurewa et al., 2023; Sophie et al.; Ali et al.2025). The Ellman method is based on the reaction between dithiobisnitrobenzoate (DTNB), or Ellman's reagent, and free sulfhydryl groups (–SH). It is an extremely useful and convenient technique widely used to characterize enzyme inhibition kinetics of enzymes presenting this functional group at their active site. (de et al.2022; Sullivan, 2023); Srinivasan, 2023). Determining the inhibitory activity of new compounds on enzymes is critical in biochemical, pharmaceutical and toxicological research. Enzyme inhibition assay methods offer a quick and efficient way to evaluate the potency of a test compound and are therefore regularly used to monitor enzyme activity profiles. Since its introduction in 1961 by George Ellman, the Ellman method has become a popular choice often used in drug discovery programs, mechanistic and toxicological studies. The procedure consists of adding Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid), DTNB) to the reaction mixture to measure the quantity of free thiol groups on biomolecules. The resulting colored product is subsequently measured spectrophotometrically at 412 nm to estimate the concentration of liberated thiols, providing an effective approach for the characterization of enzyme inhibition with great sensitivity and tolerance to the test compounds. (De et al.2022; De et al.2023; Carlsson & Luttens, 2024; McAulay et al., 2022; Lamba & Pesaresi, 2022).

1.1 Enzyme Assays and Activity Measurement

Enzyme assays provide a quantitative method for evaluating the activity of an enzyme by measuring how much of the substrate is being converted to the product of the reaction over time. The fundamental principle underlying assays for enzyme inhibition entails measuring the activity of an enzyme in the presence and in the absence of the inhibitor (Rufer, 2021; Copeland, 2023; Mons et al., 2022). Enzyme inhibitors can be broadly categorized into irreversible and reversible inhibitors. Irreversible inhibitors bind to or react with an enzyme to inactivate it. If an inhibitor can be removed from an enzyme to restore the enzyme activity, it is a reversible inhibitor. Reversible inhibitors can be further classified into competitive, non-competitive, and uncompetitive inhibitors (R. Ramsay & F. Tipton, 2017). Competitive inhibitors compete for the enzyme's active-site, thereby preventing the substrate from binding to the enzyme. In contrast, non-competitive inhibitors bind to a different location on the enzyme, thereby altering the enzyme structure so that the substrate cannot bind well. Uncompetitive inhibitors also bind to a site other than the active site, but only when the enzyme is bound to the substrate. Although the substrate can still interact with the enzyme when the inhibitor is present, the reaction cannot proceed to the formation of the product (Hollenberg, 2022; Srinivasan, 2023; Hajizadeh et

al.2022; Pérez et al.2021). For example, the Ellman method provides a continuous assay for irreversible inhibition of enzymes that cleave thioester or thioester-like substrates. In the Ellman assay, substrate cleavage releases a thiol product that reacts with a sensitive chromophore, giving a colored product that can be readily monitored by UV/Vis spectroscopy. Because the Ellman procedure provides a continuous assay of the enzyme activity, it can also be used to study reversible enzyme inhibitors (Pohanka, 2025; De et al.2022; Zhu et al.2025).

1.2 Types of Enzyme Inhibitors

Inhibitors are typically classified by their kinetic mechanism into competitive, non-competitive, or uncompetitive (R. Ramsay & F. Tipton, 2017). A competitive inhibitor raises the apparent Km for the substrate without necessarily preventing its binding, whereas partial non-competitive inhibition results in a less active complex, reducing the apparent kcat. Inhibitors that bind very tightly to an enzyme can cause inhibition at concentrations comparable to the enzyme, with slow onset and dissociation rates (Dong et al.2023; Olivieri et al.2022). Examples of such slow, tight-binding inhibitors include cimoxatone, harmaline, and methylene blue when targeting monoamine oxidase. Some enzymes exhibit high substrate inhibition, where increasing substrate concentrations beyond an optimum value decrease activity. Acetylcholinesterase (AChE), for example, has a peripheral anionic site that can bind the substrate in addition to the active site. At low-to-moderate substrate concentrations, binding to the peripheral site enhances catalysis, causing velocity to increase more than expected; however, at higher concentrations, simultaneous occupation of both sites by substrate can inhibit activity, leading to a velocity decline (Garcia-Molina et al., 2022; Baksi et al.2023; Zhang et al., 2025; Peeples & Rosen, 2021; Meng et al.2021). Thus, excessive substrate levels can produce apparent inhibition, complicating kinetic analyses. Assay interference must also be considered, as factors such as pH shifts, ionic strength changes, or contaminants can mimic inhibitory effects. In tyrosinase inhibition assays, for instance, preincubation with certain inhibitors may scatter results, and high concentrations of DMSO can cause enzyme inactivation. Cinnamate esters often act as more potent inhibitors than their parent compounds, displaying non-competitive or mixed-inhibition kinetic profiles with IC_{50} values around 2^{-10} μM. By contrast, related compounds like stilbenes containing a resorcinol moiety tend to be more inhibitory than their phenolic analogues; however, if solvents interfere or compounds behave as alternative substrates, these observations may be artifacts. Proper controls and assay design are therefore essential to discriminate genuine enzyme inhibition from mimicking phenomena (Oufensou et al.2021; Menezes & Campos, 2024; Tian et al.2025; Moreno-Robles et al.2023; Ene et al.2025; Citarella et al.2024).

1.2.1 Competitive Inhibitors

Ellman reagent (5,5-dithiobis-(2-nitrobenzoic acid)), or DTNB, withstands the reducing environment of the cytoplasm. It is a yellow disulfide used to quantify the number or concentration of thiol groups (-SH) in a sample. This reaction is the basis for the Ellman assay, a popular method to evaluate enzyme inhibition, especially with cholinesterase, or ChE. Enzymes with variety of physiological functions such a ligands, hormones, toxins and so forth. Understanding the effects of enzyme inhibitors can provide additional insight into inhibitor properties and the enzyme itself, which is the reason the Ellman's Method has attracted much attention from the pharmaceutical industry. Competitive, non-competitive and uncompetitive enzyme inhibitor are the three aforementioned classification from which the Ellman method is also able to distinguish among A competitive inhibitor restricts the binding of a substrate to the active site by mimicking the shape of the substrate (Durmaz et al.2022**;** Dăescu et al.2024**;** Maboko et al.2024**;** Neto et al.2025; Yoshino & Murakami, 2015). Both inhibitor A and substrate S necessarily compete to bond with free enzyme E. When competitive inhibitor A bonds to the free enzyme molecule, it occupies the binding site of S and prevents it from

interacting with the enzyme. As the substrate concentration increases, the effect of A diminishes and becomes ignorable, due to the competition between A and S. For this reason, the maximum reaction rate v_{max} remains completely unaffected, regardless of the value of the inhibitor concentration [A] (Li et al., 2023; Balestri et al.2022). The Ellman method enables the determination of thiol groups in a sample by reacting with the Ellman reagent to form a mixed aromatic disulfide and the compounds 2-nitro-5-thiobenzoic acid, which shows a strong absorbance signal at 412 nm. The concentration of the latter is directly related to the concentration of thiol groups in the sample. The continuous spectrophotometric assessment of the concentration of 2-nitro-5-thiobenzoic acid provides an indirect evaluation of the enzyme activity at a specific pH, temperature, or substrate concentration. During an Ellman assay, the substrate cholinesterase is transformed by the enzyme to yield thiocholine and acetic acid. The thiocholine subsequently reacts with Ellman reagent, resulting in the formation of a yellow-colored 5-thio-2-nitrobenzoate compound with a measurable absorbance at 412 nm. **(Ginet et al.2025;** Souza et al.2024**;** Cao et al.2025**;** Poimenova et al.2024**;** Shcherbatykh et al., 2022**).**

1.2.2 Non-competitive Inhibitors

The interaction of a non-competitive inhibitor (I) with an enzyme (E), with or without a substrate (S), is an important mechanism of enzyme inhibition. The substrate saturation curves of the enzyme–substrate complex (catalytic site) are greatly affected by non-competitive inhibitors. The substrate inhibition types of choline esters or lined-up dipeptides for acetylcholinesterase in the presence of non-competitive inhibitors (such as propidium, gallamine or alcuronium) were investigated using Dixon's plot and the substrate-inhibition plot. The substrate inhibition type was converted to the partial types at higher concentrations of the non-competitive inhibitors. Non-competitive inhibitors were also screened for acetylcholinesterase and butyrylcholinesterase (Pesaresi, 2023; Maulana et al.2024; Mai et al., 2021; Masson and Mukhametgalieva,2023). The enzyme activity did not approach to zero even in the presence of a large quantity of non-competitive inhibitor or substrate. The Ellman method, which is useful for assaying cholinesterase activity, was applied to these studies, monitoring thiol group formation (Gronow, 2022; Folda et al.2025; Yoshino & Murakami, 2015).

1.2.3 Uncompetitive Inhibitors

Uncompetitive inhibitors target the enzyme-substrate complex, binding to the active site (Lamba & Pesaresi, 2022). Because uncompetitive inhibitors bind only to the enzyme-substrate complex, the magnitude is dependent on substrate concentration; hence, the double reciprocal and secondary plots are curved. Inhibition plots employ the apparent values of $1/V_{max}$ and $1/K_m$ versus inhibitor concentration with the following expressions (Ellman, 2022; Yadav et al.2025; Alnoman et al., 2025).

1.3 Application of the Ellman Method

The Ellman method of enzymatic analysis uses 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), commonly known as Ellman's reagent. The compound reacts with thiol compounds to give the 2-nitro-5-thiobenzoate dianion (TNB), which is yellow and can be detected by absorption at 412 nm (Lamba & Pesaresi, 2022). In a typical assay, the enzyme (e.g., acetylcholinesterase (AChE), butyrylcholinesterase, or carboxylesterase) hydrolyzes a thiocholine ester to give thiocholine, which in turn reacts with DTNB. The reaction is monitored by a color change, from clear to yellow. Inhibition of enzymatic activity can be monitored by decreases in the rate of yellowing. Different types of inhibitors can be distinguished: competitive inhibitors cause the reaction to proceed slowly or not at all at short times, with a later rapid increase in velocity; non-competitive inhibitors reduce the reaction velocity; and uncompetitive inhibitors can, at

high concentration, cause a decrease in absorbance with time. The method is used to screen AChE inhibitors, an important step in the search for new medications against neurodegenerative diseases such as Alzheimer's (Sullivan, 2023**;** Sattar et al.2024**; Yadav et al.2025; Wu et al., 2022;** Feng et al., 2022)

1.3.1 Biochemical Studies

Biochemical studies focus on understanding biological processes at the molecular level, encompassing the structure and function of biomolecules such as nucleic acids, proteins, lipids, and small molecules. The analysis of enzyme activity and enzyme-inhibitor interactions is an essential component of these investigations, providing insight into the mechanisms underlying biological regulation (Ali et al.2024; Haddadzadegan et al.2023; R. Ramsay & F. Tipton, 2017). Enzyme inhibition represents a fundamental biochemical process involving the reduction or suppression of enzymatic activity through interactions with inhibitory molecules. The modulation of enzyme function by inhibitors is of paramount importance in the regulation of metabolic pathways and the development of pharmacological agents targeting specific enzymes, thus underscoring the broad relevance of enzyme inhibition within the fields of biochemistry and pharmacology (Poimenova et al.2024; Zaman et al., 2021).

1.3.2 Toxicology

In addition to its significance for biochemical analyses and drug development, the Ellman method is valuable for toxicological applications. Toxic insults such as organophosphate poisoning induce a reduction in acetylcholinesterase levels, the enzyme responsible for hydrolyzing the neurotransmitter acetylcholine at mammalian synapses (Paramanya et al.2024). Organophosphates may be encountered through accidental exposure to chlorpyrifos-containing pesticides or development of chemical nerve agents. Quantification of cholinesterase activity provides ample information to aid in the diagnosis and treatment of poisoning. The rate of enzyme activity in blood erythrocytes and plasma is readily measured by the method; this provides a useful technique for emergency rooms and field personnel (Souza et al.2024; G. McGarry et al., 2013).

1.4 Advantages of the Ellman Method

Several factors contribute to the popularity of Ellman's method for assessing enzymes inhibited by molecules acting at their active site, exemplified by acetylcholinesterase. A particularly useful feature of Ellman's reagent is the potential to measure positively or negatively charged substrates or products. A second advantage of the Ellman method is the presence of some certainty that the measurement of activity is due to the enzyme rather than the inhibitor. Unlike many other methods, if AChE hydrolysis is completely inhibited no product will be formed and therefore no reaction with the colorimetric reagent will take place. This aspect is particularly important when inhibitors such as organophosphates and N-benzylpyridinium derivatives are present (Jiang et al.2025; Paramanya et al.2024; Haddadzadegan et al.2023).

1.5 Disadvantage

The Ellman method also presents a number of drawbacks. Several factors can interfere with the assay, including impurities in reagents used for sample preparation and the presence of lipids in the assay mixture. These contaminants may give rise to undesirable side reactions with Ellman's reagent, resulting in elevated background levels of the 5-thio-2-nitrobenzoic acid product and consequently, ambiguities in data interpretation (CLB et al.2024; Novák et al.2021). In addition, certain enzyme inhibitors may themselves react with Ellman’s reagent and are therefore liable to further complicate data analysis. Moreover, the method’s quantitative limits can restrict its application to specific experimental requirements (Shcherbatykh et al., 2022; Lamba & Pesaresi, 2022).

1.6 Comparison with Other Methods

Spectrophotometry offers a straightforward route to elucidating enzyme-inhibitor interactions. Upon addition of Ellman's reagent to vesicular acetylcholinesterase (AChE), a linear increase in absorbance at 412 nm is observed, indicative of continued reagent consumption. This rate diminishes when AChE is pre-incubated with iso-OMPA, a selective inhibitor of butyrylcholinesterase, allowing precise quantification of inhibition by anoctamin-1 (ANO1) modulating compounds (Cerkezi et al.2024; Gronow, 2022; Lamba & Pesaresi, 2022). Fluorescence methods, while widely employed to probe enzyme-inhibitor binding with remarkable sensitivity, often exploit pH-dependent fluorescence changes arising from tryptophan protonation and can alter molecular dynamics, occasionally yielding ambiguous results. Ellman's approach, by contrast, defines enzyme inhibition through measurement of physiological product formation, conferring a specificity that complements the range of available techniques. At a minimum, Ellman's method provides an initial assay platform for membranes and solubilized systems, enabling swift, inexpensive evaluation of AChE binding properties and guiding subsequent exploratory characterization of putative inhibitors (Torlopov et al.2023; Schmidt et al.2023; Ellman, 2022; Jain et al., 2024).

1.6.1 Spectrophotometric Methods

Enzyme inhibitors reduce or halt enzyme activity, either reversibly or irreversibly. Many drugs function by inhibiting specific enzymes, such as atorvastatin (a statin) for cholesterol lowering via HMG-CoA reductase inhibition, omeprazole for acid secretion reduction by inhibiting the H+/K+-ATPase pump, and acetazolamide, a diuretic that inhibits carbonic anhydrase. Inhibiting enzymes associated with bacteria, viral or parasite infections exemplifies the pharmaceutical importance of enzyme inhibition. Therefore, identifying and studying enzyme inhibitors is central to biochemistry and pharmacology (Ellman, 2022; Torlopov et al.2023; Jain et al., 2024). The Ellman method facilitates the quantitative measurement of sulfhydryl groups in solution. Ellman's reagent, or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), selectively interacts with free thiol groups in compounds such as glutathione, producing the reduced 2-nitro-5-thiobenzoate (TNB^-). This reaction generates a yellow product whose concentration can be determined by measuring absorbance at 412 nm using UV–vis spectrophotometry (Shcherbatykh et al., 2022; Ellman, 2022; Xiao et al.2024). Enzyme inhibition studies are pivotal in pharmaceutical chemistry and biochemical research, enabling insights into enzyme reaction mechanisms and structure–activity relationships. Successful screening of enzyme inhibitors involves producing the target enzyme and assessing changes in enzymatic activity in the presence of various compounds (H. Ridgway & Harry B. Mark, 1965; G. McGarry et al., 2013). The Ellman method has widespread applications, including in pharmaceutical analysis, biodegradable ester design, biochemical research, and toxicology (Younas et al.2023; Ellman, 2022).

1.6.2 Fluorescence Methods

Enzyme inhibitors interfere with enzymes by competing for substrate bindings, by binding to the enzyme's allosteric site, or by reacting with the enzyme's active site (Lee et al., 2014). Thus, the Ellman method is valuable for probing the activities and mechanisms of enzyme inhibitors (V. Fejzagić et al., 2020). As a highly specific indicator of thiols, Ellman's method is widely used to detect free sulfhydryl groups in proteins and other biological compounds, where it has demonstrated utility in probing enzyme inhibitory activity. Ellman's reagent itself has only recently been adapted as a fluorescent probe for thiols: fluorescence methods generally offer greater sensitivity and can avoid interference from compounds that absorb at the assay wavelength (Jain et al., 2024; Schmidt et al.2023). Fluorescence probes are therefore gaining popularity as a complement to spectrophotometric methods. Numerous fluorescent Ellman-type

reagents ultimately give rise to the same colored product as the classic Ellman procedure when combined with thiols, extending the reach of the method in monitoring enzyme inhibition (Dulaney & Taylor, 2022; Younas et al.2023).

1.6.3 Case Studies

A wealth of case studies demonstrates the widespread impact of the Ellman method in pharmaceutical, biochemical, and toxicological research. In inhibitor screening, the method is widely employed to identify novel compounds exhibiting inhibition against enzymes such as acetylcholinesterase and butrylcholinesterase (Lamba & Pesaresi, 2022). Because the assay monitors free catalytic sites, it permits the study of reversible and pseudoirreversible inhibition during enzyme and inhibitor preincubation. The Ellman assay is amenable to kinetic examination of inhibition mechanisms, enabling the differentiation of slow and fast conductors and the measurement of inhibition constants from a single progress-curve without initial-rate analysis. Furthermore, the method facilitates multiple-turnover mechanistic studies of covalent enzyme modification under single-turnover conditions, providing a catalytic perspective on enzyme–inhibitor interactions and guiding the design of improved inhibitors.

1.7 Inhibitor Screening

Enzyme inhibition plays a vital role in the regulation of metabolic reactions and the elucidation of enzymatic mechanisms. Inhibitor binding can interfere with catalytic activity without necessarily causing the enzyme to dissociate from the substrate. Consequently, the quantitative evaluation of enzyme inhibition is an indispensable tool in both fundamental and applied biochemical research. Classical methods employed for measuring inhibitor effects include absorption spectroscopy, circular dichroism (CD), and fluorescence analysis. Nevertheless, these techniques often require specialized instrumentation and demonstrate limited sensitivity when applied to enzymatic reactions that yield only small spectral variations (R. Ramsay & F. Tipton, 2017). In contrast, Ellman's method, characterized by high sensitivity, specificity, and user-friendliness, is broadly utilized for inhibitor screening assays (Zhang et al., 2023). Moreover, the method is particularly well-suited for probing inhibition mechanisms in enzymatic reactions involving inaccessible substrates.

The Ellman method constitutes a widely applied colorimetric assay developed by George L. Ellman in 1959 to quantify sulfhydryl groups within proteins and other analytes. Owing to its high specificity for detecting thiol groups, the method has become a standard tool for assessing enzyme inhibition. Its core utility was originally demonstrated for the spectrophotometric determination of tissue sulfhydryl groups. At its foundation lies the chromogenic reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), also referred to as Ellman’s reagent or Ellman’s salt, which reacts with free sulfhydryl groups to yield a chromophoric product. Specifically, DTNB interacts with thiol groups (RSH) according to the reaction: RSH + DTNB → R–S–TNB + TNB^−. The liberated TNB ion exhibits a visible absorbance maximum, facilitating colorimetric analysis. Consequently, acetylcholine esterase (AChE) inhibition can be assessed by monitoring the residual quantity of the chromophoric product resulting from enzymatic cleavage of the chromogenic substrate acetylthiocholine iodide in the presence of DTNB (Tokalı et al., 2023; Mughal et al.2024; Korkmaz et al.2025; Zhao et al.2025; Rehman et al.2023; Kirubakaran et al.2024). After Ellman's initial publication, the material was commercially available within two years and has subsequently attained widespread adoption (Rinaldi et al.2025; Mughal et al.2024).

1.8 Mechanistic Studies

Characterizing enzymatic reaction mechanisms is critical to understanding biological processes, in the development of medicines, and in the design of materials. Experimental methods that monitor various physicochemical properties can be used to probe mechanism, many of which measure the time course of the enzyme reaction. (Mouawad et al.2024; Kaya et al., 2024; Liu et al., 2023). The most common approaches rely on either optical spectroscopy or mass spectrometry of the substrate and product. The spectroscopic methods commonly rely on direct observation of the substrate/product absorbance, fluorescence or luminescence under single- or dual-wavelength detection conditions. Circular dichroism, light scattering, turbidimetry, as well as other physical properties can also be used to monitor enzymatic reaction mechanisms (Li et al., 2022; Aksoy et al., 2021; Sinha et al.2022; Bedair et al., 2022). Cholinesterase and some other enzymes contain an active-site Histidine residue that must be deprotonated to serve its catalytic role. The pKa of this histidine changes upon substrate interaction. pH methods that measure absorbance or fluorescence changes or shifts in gating current can be used to detect this change, although calibrating for the kinetics of the pKa change may be problematic (Sivaraman et al.2022; Yakan et al.2022; Liu et al., 2023; D. Holmes, 1988). Alternating pH methods that fit the time-course to only a few exponentials can provide additional information. Aminoethylbenzo[b]thiophene sulfonamide, for example, has been suggested as a reference compound for pH-dependent methods, and data have been reported for Alzheimer's Therapeutics drugs that produce time-dependent inhibition of cholinesterase using this approach (Esimbekova et al., 2023; Bartolini2024; Kyi et al.2025; Lamba & Pesaresi, 2022).

1.9 Future Directions in Enzyme Inhibition Research

Investigation of enzyme inhibitors is a crucial part of drug discovery and pharmacological research (R. Ramsay & F. Tipton, 2017). The nature of inhibition may be determined from the rate vs. substrate concentration plots and it can be classified as competitive, non-competitive, and un-competitive (Lamba & Pesaresi, 2022). At varying doses of inhibitors, quantitative evaluation of enzymatic activities can be crucial for assessing the inhibitory process as well as its characteristics (Hajizadeh et al., 2022). Various modes of assays make use of Ellman's method for investigating enzyme inhibition. The relative sensitivity and specificity of the assay, and the convenience and accessibility of application are among the primary advantages of Ellman's method. The method can be used for isolated enzymes and, in some cases, for crude tissue enzymic extracts. When the substrate involved does not contain a thiol group, the assay provides an almost universal method for the rapid and specific determination of this enzyme activity. Ellman's method can be combined with other assay techniques for simultaneous determination of specific groups present in the enzyme is analyzed. Activity in multiple-enzyme systems and for the study of complex biological phenomena can be explored using these sets of analytical tools.

1.10 Ethical Implications

Laboratory practice related to enzyme inhibitors implies a series of problems often rarely discussed in the literature, even though they are crucial for the objectives of a project. This problem is particularly relevant for specific enzyme classes (such as esterases) for which the types and characteristics of inhibitors and the scope of control of the results are little known (Legerská et al.2022; Patel, 2023). The issue deserved attention also because the techniques used to study enzyme inhibition are often sophisticated and technologically advanced and discrepancies that can be easily suspected from simple observations sometimes remain undetected (Legerská et al.2022; Liu et al., 2023; Lamba & Pesaresi, 2022). Enzyme inhibition is at the basis of several pharmacological therapies; an example is compounds able to inhibit

acetylcholinesterase (AChE) which have been used for the treatment of Alzheimer's disease to increase the concentration of acetylcholine in the Central Nervous System. In this context, different problems have been found in the use of a compound (galanthamine) that is one of the three FDA-approved AChE inhibitors: kinetic models have been developed to specifically investigate (Bartolini2024; Legerská et al.2022). Those methods aimed at estimating the degree of enzyme inhibition. This discussion should be considered as an opportunity for those researchers involved in the study of enzyme inhibitors to make an assessment of the reliability of their own results, or to future scientists planning to work in this field, to be aware in advance of the possible pitfalls of the study and a few possible solutions (Bedair et al., 2022; Li et al., 2022).

1.11 Summary of Key Findings

Research at the University of Sassari outlines that initiating reactions with the enzyme yields initial velocities independent of inhibitor concentration, indicative of slow-inhibition. The formation rate of the EI complex appears directly proportional to galantamine concentration, causing a delay in reaching the inhibited steady-state. Conversely, when reactions commence subsequent to a 20-minute enzyme-inhibitor pre-incubation in the presence of substrate, progress curves reveal slow dissociation of the inhibitor (Durmaz et al.2022; Sullivan, 2023; Sivaraman et al.2022). Under these conditions, initial velocities consequently underestimate true steady-state rates. Application of the Michaelis–Menten framework to the data obtained from enzyme-initiated reactions suggests a competitive inhibition mechanism with a Ki of 122.8 nM, whereas substrate-initiated conditions denote mixed-type inhibition characterized by Kic of 18.0 nM and Kiu of 52.7 nM. The precision of inhibition-type discrimination is affected by the chosen initiation protocol, along with reagent concentrations and the extent of the progress curve included in the fit, thereby influencing the inferred potency of the inhibitor (Başar et al.2024; Kaya et al.2023; Chafaa et al.2024; Lamba & Pesaresi, 2022).

1.11.1 Recommendations for Researchers

The Ellman method is highly sensitive and displays remarkable selectivity for thiols, enabling rapid determinations of sulfhydryl groups generated by enzymatic reactions. Although widely employed for preliminary enzyme-inhibition screening, evidence indicates that some compounds with promising inhibitory activity in Ellman screens lose potency when subsequently evaluated via classical spectrophotometric assays using natural substrates (Alasgarova et al.2025; Bedair et al., 2022; Lamba & Pesaresi, 2022). Consequently, while the Ellman method offers a facile and expedient means of detecting enzyme inhibition upon discovery, corroboration with biologically oriented techniques is advisable to validate bioactivity. (Kyi et al.2025; Sinha et al.2022).

2. CONCLUSION

The Ellman method remains a valuable tool for investigating enzyme inhibition due to its sensitivity and simplicity. The technique detects the formation of thiol groups and can monitor the hydrolysis of thioesters and disulfide bonds (Lamba & Pesaresi, 2022). In contrast, other approaches detect the consumption of substrates or cofactors directly. Because the method focuses on the formation of reactive thiol groups rather than a substrate or cofactor, it provides an improved signal in many cases. This characteristic is especially useful for following the kinetics of enzymes such as cholinesterases, cystathionine β-synthase, and thioesterases that liberate thiol groups during catalysis. Despite its advantageous features, the Ellman method is not without limitations. Interference from compounds containing thiol groups or other reactive species can lead to false positives or negatives, complicating data interpretation. The method's

quantification capacity is contingent on the absence of such interfering substances and the calibration of assay conditions. Researchers should therefore consider the chemical environment of their assay systems when employing Ellman-based measurements.

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