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Analytical aspects of enzyme reversible inhibition

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ABSTRACT

A simple graphical method for the determination of reversible inhibition type, inhibition constant (K_i) and estimation of fifty percent of inhibition I_{50} of an enzyme reaction is described. The method consists of plotting experimental data as "degree of inhibition" versus the inhibitor concentration at two or more concentrations of substrate. Diagnosis of inhibition type is based on determination of I_{50} and the observation of the shift of the inhibition curves. Relationship between I_{50} and inhibition constant K_i was discussed. A simplified hyperbolae equation of degree of inhibition showing kinetic orders of 1 and zero at low and high concentrations of inhibitors respectively is proposed. The relative error of inhibitor concentration increased drastically when degree of inhibition reached values of 90%. Examples of published inhibition reports as well as an experimental example of amperometric biosensor based on tyrosinase inhibition by benzoic acid were in agreement with the proposed theoretical approach.

1. Introduction

Reversible enzyme inhibition requires graphical plots for diagnostic of inhibition type and for the determination of inhibition constant K_i. Hence, Lineweaver–Burk [1], Dixon [2] and Cornish-Bowden [3] were proposed, but none of these plots alone give satisfactory results. During several years, it was very common to use the Dixon's representation to determine the inhibition type and the inhibition constants for competitive and noncompetitive inhibitions [2]. However, this method is unable to distinguish between competitive and mixed inhibition types. The Cornish-Bowden method is an improved method for determining inhibition constant of uncompetitive inhibition [2], but it is not able to determine the inhibition constant for competitive inhibition. Indeed, secondary plot of Lineweaver–Burk representation or the use of both of Dixon and Cornish-Bowden plots are widely reported in biochemical research laboratories in order to solve the problem of determination of type of inhibition and K_i.

Also I_{50} (the concentration of inhibitor which causes 50% inhibition) is commonly used in pharmacological practice. This I_{50} is often regarded as a value simply equal to K_i but that is only true in the case of noncompetitive inhibition. In order to improve human health, many drugs based on enzyme inhibition have been evaluated, demonstrating the importance of enzyme inhibitors. I_{50} is essential for describing the extent of inhibition, in order to study the effect of drugs on enzymes. In other words, it is very useful to compare the values of I_{50} measured in different laboratories for the same substrate and enzyme, to assess the effectiveness of inhibitory compounds. The pharmacological treatment of some diseases is currently based on enzyme inhibitors like cancer, diabetes type II and neurologic disorders [4–6]. The relationship between the inhibition constants K_i and I_{50} for competitive, uncompetitive and noncompetitive inhibition was discussed [7,8]. Plots reporting the degree of inhibition versus concentration of inhibitor are often found in papers dealing with bioassays and biosensors based on enzyme inhibition. Although the huge number of papers are published in this field [9–15], the study of analytical aspects of enzyme inhibition is scarce.

In this work, we applied the "degree of inhibition" plot for the determination of I_{50} and the type of inhibition. We propose a simplified equation, valid for all types of inhibition, for the estimation of dynamic range and relative error of inhibitor concentration. A practical example of inhibition of tyrosinase biosensor by benzoic acid is discussed.

2. Materials and methods

2.1. Materials

Tyrosinase (EC 1.14.18.1) from mushroom (3933 units mg) was purchased from Sigma. All reagents were analytical grade and were available from Sigma.

2.2. Instrumentation

Amperometric measurements were performed with a PalmSens potentiostat interfaced to a computer. A10 mL electrochemical cell

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with a conventional three electrode system consisting of carbon paste electrode as the working electrode, a platinum wire as the auxiliary electrode, and an Ag/AgCl electrode as the reference electrode were used.

2.3. Application on biosensor based on tyrosinase

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase enzyme that catalyzes the conversion of phenolic substrates to catechol and the successive oxidation to quinone [16]. Thus, the quinone can be electrochemically reduced to allow amperometric detection of phenol

$$\text{Phenol} + \text{Tyrosinase (O2)} \rightarrow \text{Catechol}$$

$$\text{Catechol} + \text{Tyrosinase (O2)} \rightarrow \text{O-quinone} + H_2O$$

$$\text{O-quinone} + H^+ + 2e^- \rightarrow \text{Catechol}$$

2.4. Immobilization of tyrosinase

A biosensor based on the immobilized tyrosinase (Tyrs) enzyme is described for the detection of catechol. The immobilization was prepared by using the cross-linking immobilization. The enzyme solution was first prepared by mixing 15 μL of Tyrs (104 unit/μL), 7.5 μL of BSA (1%) and 7.5 μL of glutaraldehyde (0.25%). The mixture was spread on the surface of a carbon paste electrode, and then it was dried at room temperature. Before use, the enzyme electrode was placed under stirring for 10 min with buffer solution to remove enzyme not firmly immobilized. After use, the biosensor was stored in phosphate buffer solution overnight at 4 °C.

2.5. Enzyme activity assays

Tyrosinase activity was measured by injecting different concentrations of catechol as substrate and by using amperometric measurement. The assays of the enzyme activity were performed in an electrochemical cell containing 10 mL of 0.1 mol l^{-1} phosphate buffer, pH 7.0 at 25 °C. The applied potential was fixed to 0 V.

2.6. Competitive inhibition by benzoic acid

To perform each measurement, the carbon paste electrode modified with Tyrs was dipped into the electrochemical cell containing 10 mL of 0.1 mol l^{-1} phosphate buffer solution (pH 7.0) maintained under constant magnetic stirring. The applied potential was fixed to 0.0 V. Once the baseline was established (15 min approximately), a defined concentration of catechol was added to the measuring cell. A large reduction current was observed due to the addition of catechol. The addition of benzoic acid solution caused a decrease in current.

3. Results and discussion

3.1. Relationship between I_{50}, K_i and concentration of substrate

Inhibitors can bind to enzymes following the expressions shown in Scheme 1 [17].

In the case of competitive inhibition (Scheme 1a), the inhibitor binds to free enzyme with a greater affinity than to the EI complex.

Eq. (1) below represents the Michaelis–Menten equation in absence of inhibitor

$$V_0 = \frac{V_{max}[S]}{K_m + [S]} \tag{1}$$

V_{max} = Maximum velocity.
V_0 = Velocity in the absence of the inhibitor.
K_m = Michealis constant of the substrate(S).
$[S]$ = Substrate concentration.

Eq. (2) below represents the Michaelis–Menten equation in presence of competitive inhibitor

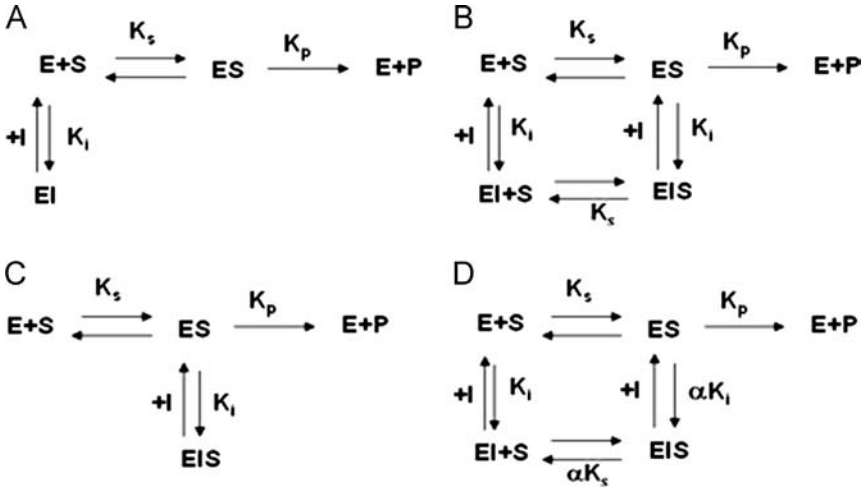
$$V_i = \frac{V_{max}[S]}{K_m(1 + [I]/K_i) + [S]} \tag{2}$$

V_i = Velocity in the presence of inhibitor.
$[I]$ = Inhibitor concentration.
K_i = Dissociation constant of EI.
When $I = I_{50}$, and $V_0 = 2V_i$, then

$$\frac{V_{max}[S]}{K_m + [S]} = \frac{2V_{max}[S]}{K_m(1 + [I_{50}]/K_i) + [S]}$$

By rearrangement

$$\frac{I_{50}}{K_i} = \left(1 + \frac{[S]}{K_m}\right) \tag{3}$$



Scheme 1. . Scheme of enzyme inhibition in case of reversible inhibition: competitive (a), non-competitive (b), un-competitive (c) and mixed type inhibitions (d).

Table 1
Summary of reversible inhibition.

	Competitive	Noncompetitive	Uncompetitive	Mixed $\alpha=2$
V_i	$\frac{V_{max}[S]}{K_m(1+[I]/K_i)+[S]}$	$\frac{V_{max}[S]}{K_m(1+[I]/K_i)+[S](1+[I]/K_i)}$	$\frac{V_{max}[S]}{K_m+[S](1+[I]/K_i)}$	$\frac{V_{max}[S]}{K_m(1+[I]/K_i)+[S](1+[I]/\alpha K_i)}$
$\frac{I_{50}}{K_i}$	$\left(1+\frac{[S]}{K_m}\right)$	1	$\left(1+\frac{K_m}{[S]}\right)$	$\alpha\left(\frac{1+[S]/K_m}{\alpha+[S]/K_m}\right)$
$y=\frac{V_0-V_i}{V_0}$	$\frac{[I]}{[I]+K_i\left(1+\frac{[S]}{K_m}\right)}$	$\frac{[I]}{[I]+K_i}$	$\frac{[I]}{[I]+K_i\left(1+\frac{K_m}{[S]}\right)}$	$\frac{[I]}{[I]+\alpha K_i\left(\frac{1+[S]/K_m}{\alpha+[S]/K_m}\right)}$
$y=V_0-V_i/V_0$	$[I]/[I]+I_{50}$	$[I]/[I]+I_{50}$	$[I]/[I]+I_{50}$	$[I]/[I]+I_{50}$
$1/y=V_0/V_0-V_i$	$1+I_{50}(1/I)$	$1+I_{50}(1/I)$	$1+I_{50}(1/I)$	$1+I_{50}(1/I)$
Shift of the inhibition curves $y=f(I)$, with increasing $[S]$	To the right	Unchanged	To the left	Slightly to the right
Ratio $\frac{I_{50([S]=10K_m)}}{I_{50([S]=K_m)}}$	5.5	1	0.5	1.5
Ratio $\frac{I_{50([S]=0.1K_m)}}{I_{50([S]=K_m)}}$	0.55	1	5.5	0.79
I	$\frac{yI_{50}}{1-y}$	$\frac{yI_{50}}{1-y}$	$\frac{yI_{50}}{1-y}$	$\frac{yI_{50}}{1-y}$
$\Delta I/I$	$\frac{\Delta y}{y}\frac{1}{1-y}$	$\frac{\Delta y}{y}\frac{1}{1-y}$	$\frac{\Delta y}{y}\frac{1}{1-y}$	$\frac{\Delta y}{y}\frac{1}{1-y}$

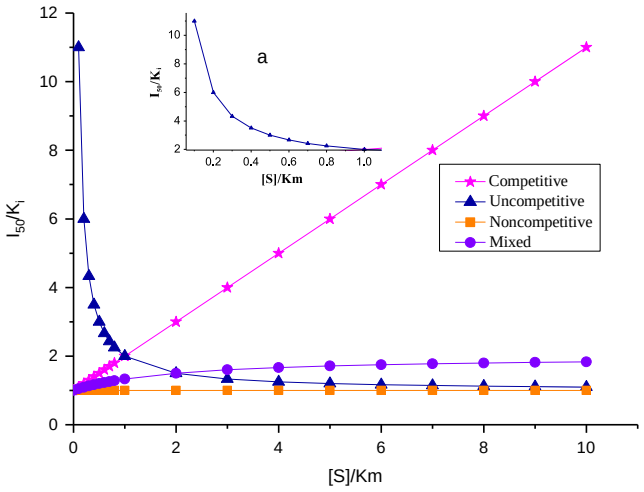


Fig. 1. Typical plots of I_{50}/K_i versus $[S]/K_m$ for various types of inhibition.

The Eq. (3) is identical to that described by Cheng and Prusoff [7]. Similary I_{50}/K_i was calculated for the other types of inhibition and reported in Table 1. Mixed inhibition was not studied by Cheng and Prusoff [7]. The demonstration of the equations obtained in the case of other type of inhibition is described in Supplementary material 1.

Fig. 1 shows the relationship between I_{50} presented as I_{50}/K_i and the concentration of substrate presented as $[S]/K_m$. The substrate concentration varied from $0.1K_m$ to $10K_m$. For the clarity of the figure, to the inhibition constant was attributed the value 1.

The analysis was presented in adimensional form, which provides a better understanding of the relationship between I_{50} and substrate concentration regardless of the absolute value of K_i, $[S]$ and K_m.

From Fig. 1 we can see clearly that in competitive inhibition, the I_{50} increases proportionally with the substrate concentration. In the case of uncompetitive inhibition, we note that the I_{50} decreases when the substrate concentration increases for the entire range of substrate concentration studied, particularly when $[S]<K_m$ as indicated in Fig. 1a. In the case of mixed inhibition ($\alpha=2$), a slight variation of I_{50} was observed. However, I_{50} was constant whatever the concentration of substrate used, in the case of noncompetitive inhibition.

These results clearly indicate without doubts that the values of I_{50} cited in research works should be accompanied with the value of substrate concentration.

3.2. Simplified equation of degree of inhibition

The "degree of inhibition", called in this work as "y", is defined by the following equation:

$$y=\frac{V_0-V_i}{V_0} \tag{4}$$

where V_0 represents the Michaelis–Menten equation in absence of inhibitor, and V_i represents the Michaelis–Menten equation in presence of inhibitor.

Case of mixed inhibition

$$V_i=\frac{V_{max}[S]}{K_m(1+[I]/K_i)+[S](1+[I]/\alpha K_i)} \tag{5}$$

By combining Eqs. 1, 4 and 5, one obtains

$$y=\frac{[I](\alpha K_m+[S])}{\alpha K_i(K_m+[S])+[I](\alpha K_m+[S])}$$

By rearrangement of equation above

$$y=[I]/[I]+\alpha K_i(K_m+[S])/\alpha K_m+[S]$$

$$y=[I]/[I]+\alpha K_i(1+[S]/K_m/\alpha+[S]/K_m) \tag{6}$$

Since $I_{50}=\alpha K_i\left(\frac{1+[S]/K_m}{\alpha+[S]/K_m}\right)$ Eq. (6) can be simplified to

$$y=\frac{[I]}{[I]+I_{50}}. \tag{7}$$

Identical equations were obtained with other type of inhibition (demonstration is shown in supplementary material 2).

The equation above represents a hyperbola.

When $[I]\ll I_{50}$, $[I]$ is neglected versus I_{50}, Eq. (7) may be simplified to

$$y=\frac{[I]}{I_{50}}$$

which refers to an equation of order 1. From analytical point of view, this means that there is linear relation between degree of inhibition and inhibitor concentration.

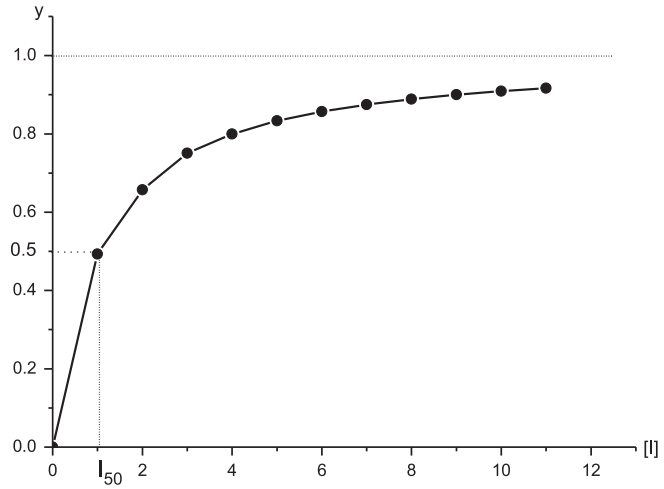


Fig. 2. General plot of degree of inhibition (y) versus inhibitor concentration.

When $[I] \gg I_{50}$, I_{50} is neglected versus $[I]$, Eq. (7) may be simplified to

$$y = 1$$

which refers to an equation of order 0. In this case, large variation of inhibitor concentration produces slight variation of degree of inhibition.

The equation is very similar to the equations of the Michaelis–Menten.

Fig. 2 shows the effect of inhibitor concentration on the "degree of inhibition". From this plot it is clear that the "degree of inhibition" increases with increasing concentration of inhibitor. The upper limit of linear range is suspected to be I_{50} unless some diffusional resistance occurs in the case of immobilized enzyme, where the transfer of inhibitor could be prevented and thus his linear range extended to high inhibitor concentration.

3.3. Determination of I_{50}

I_{50} may be directly estimated from the curve of degree of inhibition versus inhibitor concentration as indicated above in Fig. 2. Otherwise, if we look deeply in the simplified Eq. (7) proposed in this work for inhibition study, we found it very similar to the known equation of the Michaelis–Menten (1) where K_m, substrate concentration at which the reaction rate is half of maximal velocity is replaced by I_{50}, concentration of inhibitor that causes 50% of inhibition. A double reciprocal plot ($1/V$ versus $1/[S]$) is graphical representation widely used for determination of K_m. This brought us to propose double reciprocal plot ($1/y$ versus $1/[I]$) for determination of I_{50}. The inverse of the "degree of inhibition" can be directly calculated from Eq. (7) obtaining the following equation:

$$\frac{1}{y} = I_{50}(1/[I]) + 1 \tag{8}$$

The above equation is valid for all inhibition types. This equation predicts a linear relationship between $1/y$ and $1/[I]$ with the slope equal to I_{50} (Fig. 3).

The Eq. (7) may be rearranged into

$$y = \frac{1}{(1 + (I_{50}/[I]))} \tag{9}$$

For each degree of inhibition, the ratio $I_{50}/[I]$ is constant and can be calculated according to the above Eq. (9). This generates a

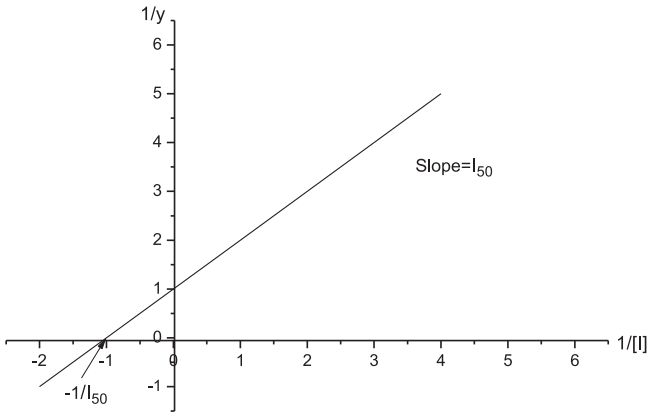


Fig. 3. Typical plot of inverse degree of inhibition versus inverse of inhibitor concentration.

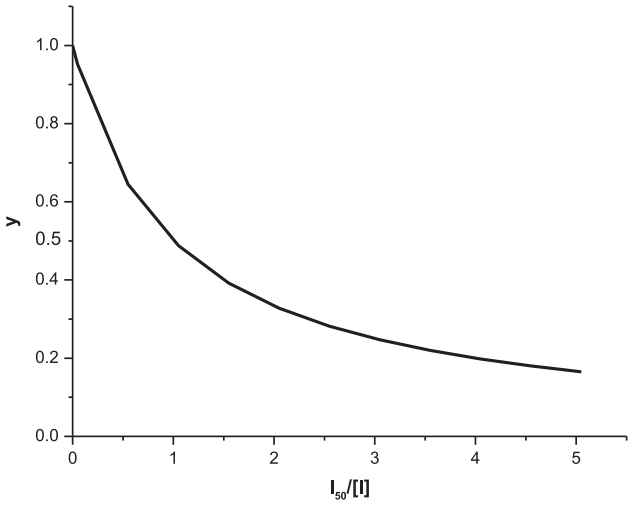


Fig. 4. Working curve of degree of inhibition versus ratio $I_{50}/[I]$.

working curve of degree of inhibition versus the ratio $I_{50}/[I]$ as indicated in Fig. 4. The corresponding values around 50% (25–75%) could be used for rapid estimation of I_{50} (Table 2). This means that a single experiment of inhibition is sufficient for an estimation of I_{50} without any prior knowledge of substrate concentration, K_i or even type of inhibition. For example, if $y = 0.4$, the expected value of $I_{50}/[I]$ is 0.714 (Table 2), so I_{50} can be calculated easily for known concentration of inhibitor. We expect that this equation and their calculated values (Table 2) will be very helpful for researcher in inhibition study.

3.4. Diagnosis of inhibition type

By estimating I_{50}, we can check the inhibition type from the "degree of inhibition" (y). Indeed, we will vary the substrate concentration to determine its effect on the I_{50} and after this change we can directly determine the inhibition type.

In the case of competitive inhibition

$$\frac{V_0 - V_i}{V_0} = \frac{[I]}{[I] + K_i(1 + [S]/K_m)}$$

and

$$\frac{I_{50}}{K_i} = \left(1 + \frac{[S]}{K_m}\right)$$

as described above in Eq. (3).

Varying the concentration of substrate from $1K_m$ to $10K_m$ causes an increase of I_{50}/K_i from 2 to 11 in agreement with Eq. (3), and thus ratio $I_{50([S]=10K_m)}/I_{50([S]=K_m)}$ is equal to 5.5 as reported in Table 1.

Fig. 5A, shows the effect of different substrate concentrations on I_{50}. For the clarity of the figure, to the inhibition constant K_i was attributed the value 1. As can be observed from the graph related to the competitive inhibition, when the substrate concentration increases, I_{50} increases too in agreement with literature [7,18], and the inhibition curve shift from the left to the right. On the contrary, the inhibition curve shifts from the right to the left when substrate increases in the case of uncompetitive inhibition (Fig. 5B), and remain unvaried in the case of non-competitive inhibition (Fig. 5C). However, slight shift from the left to the right was obtained in the case of mixed inhibition (Fig. 5D). Using the equations of I_{50} reported in Table 1 it is possible to demonstrate that increasing substrate from $[S]=K_m$ to $[S]=10K_m$correspond to an increase 5.5 times of I_{50} in the case of competitive inhibition, and only 1.5 times of I_{50} in the case of mixed inhibition. In the light of these results, one can distinguish easily the competitive, non competitive, uncompetitive and mixed inhibitions. Since the type of inhibition and the I_{50} can be determined as described above, the inhibition constant K_i can be also estimated from the equations in Table 1. An advantage of this procedure is the ability to determine the type of inhibition and K_i for an enzyme when K_m is already known. It has also the advantage to predict new value of I_{50} for other concentrations of substrate without knowing K_i. This allows an easy comparison among data from different laboratories.

Table 2
Calculated values of $I_{50}/[I]$ for the given values of degree of inhibition.

y	$I_{50}/[I]$	y	$I_{50}/[I]$	y	$I_{50}/[I]$
0.25	0.800	0.4c2	0.704	0.59	0.629
0.26	0.794	0.43	0.699	0.60	0.625
0.27	0.787	0.44	0.694	0.61	0.621
0.28	0.781	0.45	0.690	0.62	0.617
0.29	0.775	0.46	0.685	0.63	0.613
0.30	0.769	0.47	0.680	0.64	0.610
0.31	0.763	0.48	0.676	0.65	0.606
0.32	0.758	0.49	0.671	0.66	0.602
0.33	0.752	0.50	0.667	0.67	0.599
0.34	0.746	0.51	0.662	0.68	0.595
0.35	0.741	0.52	0.658	0.69	0.592
0.36	0.735	0.53	0.654	0.70	0.588
0.37	0.730	0.54	0.649	0.71	0.585
0.38	0.725	0.55	0.645	0.72	0.581
0.39	0.719	0.56	0.641	0.73	0.578
0.40	0.714	0.57	0.637	0.74	0.575
0.41	0.709	0.58	0.633	0.75	0.571

3.5. Error calculation

We are also interested in the error calculation to assess the relative error associated with inhibitor concentration.

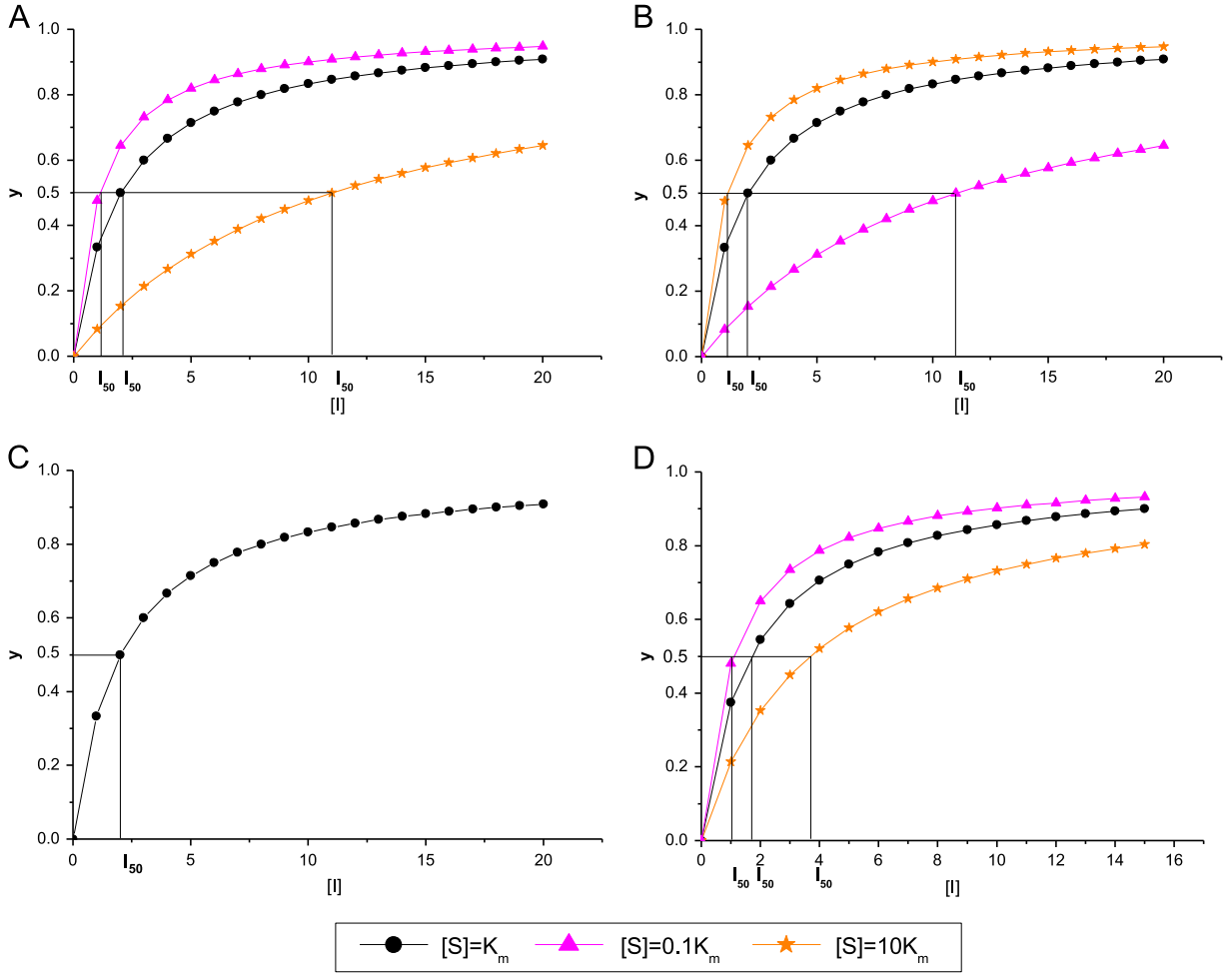


Fig. 5. Degree of inhibition plot (A) competitive, (B) uncompetitive, (C) noncompetitive and (D) mixed. The hyperbole was plotted according to Eq. (9). The following values of parameters were used: $K_i=1$ for all types of inhibition and $\alpha=2$ for mixed inhibition.

We will calculate $\Delta I/I$ and $\Delta y/y$ assuming that $\Delta V_0/V_0 \approx \Delta V_i/V_i \approx \Delta V/V = 1$, 2, 3, 4 and 5%.

Starting from the following equation of degree of inhibition

$$y = \frac{V_0 - V_i}{V_0}$$

However,

$$\frac{\Delta y}{y} = \sqrt{\left(\frac{\Delta V_i}{V_i}\right)^2 + \left(\frac{\Delta V_0}{V_0}\right)^2} \quad \text{and} \quad \frac{\Delta V_0}{V_0} \approx \frac{\Delta V_i}{V_i} \approx \frac{\Delta V}{V}$$

$$\frac{\Delta y}{y} = \sqrt{2}\,\frac{\Delta V}{V} \tag{10}$$

By rearranging the general Eq. (7) of degree of inhibition:

$$y = \frac{[I]}{[I] + I_{50}}$$

we get

$$[I] = \frac{yI_{50}}{(1-y)} \tag{11}$$

First, we calculate

$$\mathrm{Ln}\, I = \mathrm{Ln}(yI_{50}) - \mathrm{Ln}(1-y)$$

$$\frac{dI}{I} = \left(\frac{1}{y} + \frac{1}{1-y}\right)dy$$

$$\frac{dI}{I} = \left(\frac{1}{y(1-y)}\right)dy$$

$$\frac{dI}{I} = \frac{dy}{y}\frac{1}{1-y}$$

However,

$$\frac{\Delta I}{I} = \frac{\Delta y}{y}\frac{1}{1-y} \tag{12}$$

Note that $\Delta I/I$ is the same for all types of inhibition.

Fig. 6 shows the variation of relative error $\Delta I/I$ versus the degree of inhibition in the range studied (0.05–0.95) for enzymatic reaction rate varied from 1% to 5%. The relative error $\Delta I/I$ remains almost stable when the degree of inhibition y is not higher than 0.5, then starts to increase significantly reaching values higher than 20% when y is higher than 0.9. These results are in agreement with the hyperbolic curve of the inhibition (Fig. 2). Indeed, with a high degree of inhibition ($y > 0.9$) the inhibition reaction tends to be order zero with respect to the inhibitor concentration and thus the error increase. These results brought us to define the dynamic range of an inhibited enzyme reaction and to make comparison with dynamic range of uninhibited enzymatic reaction.

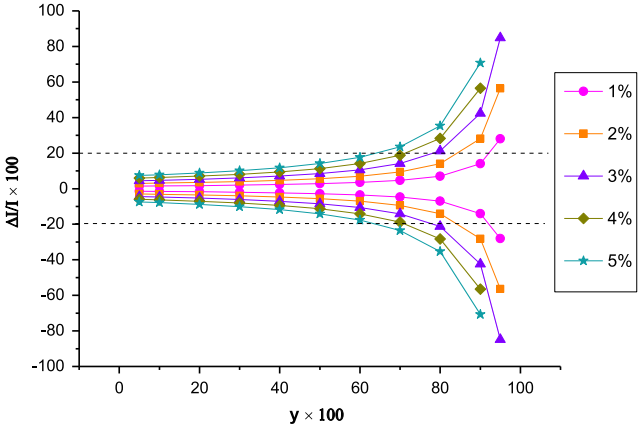


Fig. 6. $\Delta I/I$ versus the degree of inhibition (y) at various values of $\Delta V/V$ from 1% to 5%.

In the case of uninhibited enzymatic reaction, when $[S] = 0.1K_m$, $V_0 = 0.091V_{max}$ and when $[S] = 10K_m$, $V_0 = 0.91V_{max}$ in agreement with Eq. (1). Thus, the dynamic range of substrate concentration is at least two decades. Extended dynamic range to low concentration of substrate $[S] < 0.1K_m$ can be observed when a blank reagent shows low noise.

In the case of an inhibited enzymatic reaction, when $[I] = 0.1I_{50}$, $y = 0.091$ and when $[I] = 10I_{50}$, $y = 0.91$ that is in agreement with the Eq. 10. Here also the dynamic range of inhibitor concentration can be considered as two decades. However, the determination of low inhibitor concentration $[I] < 0.1I_{50}$ leads to non reproducible values of y. Indeed, V_i should be statistically different from V_0. Because V_0 represents a high response signal of the activity of the enzyme, its absolute error is also high, and thus a small variation of $V_0 - V_i$ is difficult to precisely quantify. In fact it was previously reported that 10% of inhibition ($y = 0.1$) is considered as limit of detection [12].

From Fig. 6 we can clearly see that if we considered that the confidence interval should not exceed 20% ($\Delta I/I \approx 20\,\%$), the upper limit of dynamic range is 65% ($y = 0.65$) and 93% ($y = 0.93$) when the relative error of enzymatic reaction rate $\Delta V/V$ is equal to 5% and 1% respectively.

3.6. Examples from literature

The applications of the representation of the degree of inhibition versus the inhibitor concentration and their related equations described in this work may be illustrated with some examples taken from literature (Table 3). The type of inhibition of the three enzymes resulted with our approach is similar to those reported in literature. Moreover, the curve of degree of inhibition shifts to the right for acetylcholinesterase and hexokinase as predicted by competitive inhibition and remains unvaried for the enzyme protease as in the case of non competitive inhibition. K_i was determined from the equation of the relationship between I_{50} and K_i. The close agreement between these randomly selected publications and our calculated values indicates the validity of our theoretical approach.

3.7. Experimental example

3.7.1. Amperometric biosensor based on inhibition of immobilized enzyme

In this present work, a reversible amperometric biosensor based on the immobilization of tyrosinase was applied. The principle of the amperometric biosensor was based on the measurement of the current produced when catechol is reduced by Tyrosinase at a constant applied potential.

Amperometric responses of the tyrosinase immobilized on carbon paste biosensor were investigated by consecutively increasing the concentration of catechol at a working potential of 0.0 mV allowing determination of $K_m = 52\ \mu M$.

Benzoic acid is an inhibitor of tyrosinase. The concentration of the catechol was fixed at 50 μM and 200 μM, in order to study the range of $[S] = K_{mapp}$ and $[S] = 4K_{mapp}$. Calibration curves, obtained at carbon paste electrode modified by Tyrs, were constructed by plotting the degree of inhibition against the concentration of benzoic acid. It was observed that the degree of inhibition (%) of benzoic acid increases with the increase of its concentration (Fig. 7). As we can see from the plot, when the concentration of catechol is equal 50 μM I_{50} was equal to 639 μM. However, when the catechol increases up its concentration to 200 μM, a shift of the

Table 3
Literature values versus calculated K_i and inhibition type.

Enzyme	Substrate	K_m	Inhibitor	Reported Ki, inhibition type	Calculated Ki, inhibition type	Reference
Protease (human Y-secretase)	Recombinant protein	0.15 μM	Sulfonamide	0.14 μM, non competitive	0.14 μM, non competitive	[19]
Acetylcholinesterase	Acetylcholine	77.2 μM	Galantamine	0.22 μM, Competitive	0.28 μM Competitive	[20]
Hexokinase	ATP	1.2 mM	Glucose-6-phosphate	11.5 mM, competitive	12.7 mM, competitive	[21]

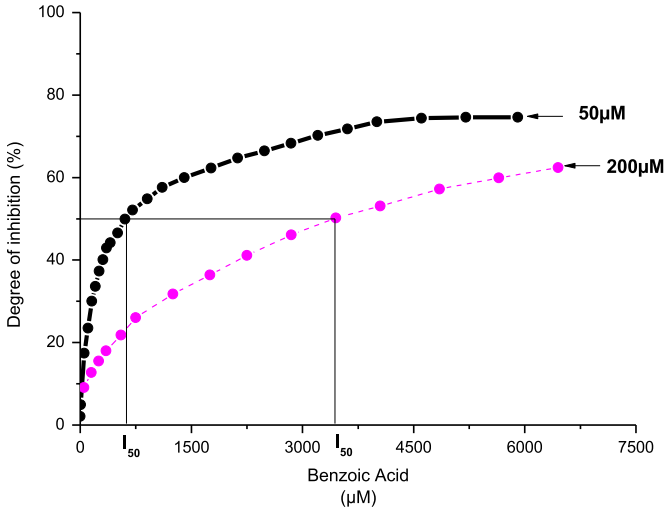


Fig. 7. Competitive inhibition of tyrosinase immobilized on the carbon paste by benzoic acid in the presence of 50 μM and 200 μM catechol in 0.1 M phosphate buffer, pH 7.0. The applied potential is 0.0 V versus Ag/AgCl.

inhibition curve to the right with an increase of I_{50} to 3439 μM was observed. Moreover, on the base of these results, it was concluded that the inhibition is competitive, in agreement with literature [22]. Taking into account the Eq. (3) and the concentrations of substrate$[S]=K_{mapp}$ and $[S]=4K_{mapp}$, the calculated increase of I_{50} ratio $I_{50([S]=4K_m)}/I_{50([S]=K_m)}$ should be 2.5 time if the enzyme is free. However, under our experimental conditions the increase of I_{50} was 5.4 time. The high value of I_{50} observed might be due to diffusional limitations of substrate through enzymatic membrane. Indeed, it is known that the values of I_{50} and K_i as well as K_m and V_{max} can change slightly due to the heterogeneous enzyme kinetic [23].

4. Conclusion

In conclusion, we describe here an easy method for the determination of reversible inhibition type and estimation of I_{50} as well as K_i of an enzyme reaction. Relationship between I_{50} and K_i were also discussed, in order to understand the difference of results reported in literature by taking into consideration the concentration of substrate employed.

The curves of degree of inhibition versus inhibitor concentration of all types of inhibitions followed the same general hyperbolae equation, where I_{50} is highlighted and can be easily determined.

It was demonstrated that the relative error of inhibitor concentration increased at $[I] \gg I_{50}$.

The proposed study of inhibition study was applied successfully to several reports cited in literature and to an experiment with biosensor based on enzyme inhibition.

We hope that this method will find widespread acceptance in a scientific community for the monitoring of inhibitors in clinical, pharmaceutical, environmental and food laboratories

Appendix A. Supplementary mateials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.10.025.

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