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Dedicated to professor Gh. Marcu at his 80th anniversary

INHIBITORY EFFECT OF METOPROLOL UPON CATALASE–H_2O_2 DECOMPOSITION, USED AS A POTENTIAL KINETIC METHOD TO DETERMINE THE DRUG CONCENTRATION

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ABSTRACT. The enzyme catalyzed process of decomposition of hydrogen peroxide has been investigated by means of a Clark oxygen sensor, in the presence and absence of various concentrations of Metoprolol – a β-blocker drug - having an inhibiting role. The Michaelis – Menten kinetic parameters of H_2O_2-catalase reaction have been determined from Lineweaver-Burk plots. The inhibition pattern we have deduced, suggested by the Lineweaver - Burk plots, corresponds to a fully mixed inhibition mechanism. Inhibition constants K_I and K_I' were determined. The inhibitory effect can be used to determine metoprolol in low concentrations by a kinetic method.

Keywords: Metoprolol, Catalase, Hydrogen peroxide decomposition, Inhibition mechanism, Kinetic methods of analysis.

INTRODUCTION

Inhibitors of enzymatic reactions have acquired large applications in medical and pharmaceutical research [1]. The catalase mediated decomposition of H_2O_2:

$$2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2 \quad (1)$$

has been the subject of extensive investigations both with regard to the kinetics and to the mechanism of the reactions.[2, 3, 4, 5]

Similar reactions were also observed for other organic peroxides [6, 7]
Well-known inhibitors of these reactions are ions like azide, cyanide and fluoride [8, 9, 10], or some organic compounds like 3-amino-1, 2, 4-triazole [11] atenolol [12] and metoprolol [13].

Catalase is widely distributed in nature. It is found in all aerobic microorganisms, in plant and animal cells [14]. The catalase activity of mammalian tissues varies greatly: it is highest in liver and kidney and low in connective tissues. The enzyme, when located in organelles, acts as a regulator of the H_2O_2 level, while in erythrocytes, catalase provides protection for hemoglobin against the oxidizing agents like H_2O_2 together with gluthathione peroxidase [16].

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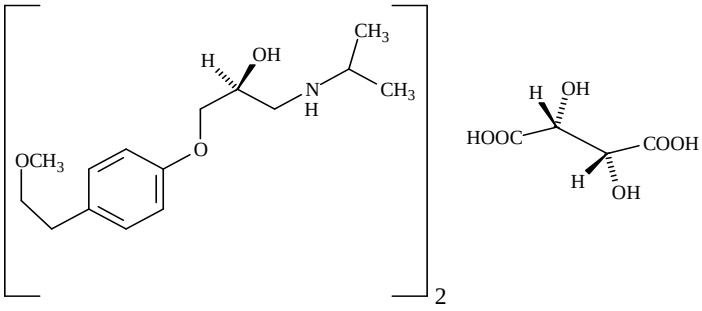
Inhibitors are compounds that slow down the activity of the enzyme by preventing either the formation of substrate-enzyme or breaking down enzyme-product complexes.

Metoprolol ($C_{34}H_{56}N_2O_{12}$) is a cardioselective β-adrenoceptor blocking agent (β-blocker). Its formula is (±)-1-isopropyl-amino-3-p-(2-methoxyethyl)- phenoxypropan-2-ol(2R,3R)-tartrate with number 56392-17-7, in accordance to British Pharmacopeia[17],



This agent is used in clinical medicine for the treatment of various diseases including hypertension, pectoral angina, cardiac aritmia, and especially ventricular tahicardic and hart attract, [18, 19, 20]

A number of analytical methods have been developed for its determination including spectrophotometrical [21], potentiometrical and amperometric methods [22, 23, 24]. The purpose of this work is to establish the type of inhibition for this reversible inhibitor by it effect on the rate of hydrogen peroxide decompositions catalyzed by catalase.

EXPERIMENTAL

Equipment. The measurements were undertaken with a Clark oxygen sensor, attached to a Multiline P4 multimeter with automatic data acquisition on a PC. The sensors cover a measuring range from 0 up to 19.99 mg/L for the dissolved oxygen, with a resolution of 0.01 mg/L and an accuracy of ±0.5% from 5.30 ℃. All experiments were performed in a vessel provided with a water jacket. To maintain a constant temperature value (20±0.1 ℃) it was connected to a Falc 90 rec irculation water bath. The reaction mixture was stirred during the run with a magnetic stirrer, always with the same frequency.

Reagents and solutions. We used bacterial catalase from *Micrococcus Lysodeikticus* 176340 U/ml (where the enzyme unit 1U is the amount of enzyme needed to transform 1 μmol of the substrate within 1 min, under standard conditions), from FLUKA with a purity index of 0.85, which came from the ratio of absorbance at 405 nm and 280 nm, A_{405}/A_{280}.The molar concentration of catalase was determined spectrophotometrically at 407 nm, where the molar absorptions coefficient is known to be $4x10^5$ mol^{-1} dm^3 cm^{-1} [21].

All the other reagents were of analytical reagent grade. The solutions were prepared with de-ionized, four-times distilled water in order to avoid the interference of heavy metals. Stock solutions of catalase ($9x10^{-10}$ mol/L), H_2O_2 (4.9×10^{-2} mol/L) and metoprolol (10^{-5} mol/L) in phosphate buffer of pH=7.0 were freshly prepared before

each set of runs. Hydrogen peroxide has been standardized against potassium permanganate in acidic media.

Procedure. The reaction mixture of 10 mL volume was prepared directly in the reaction vessel connected to a thermostat. All the solutions of the reagents were kept in the water bath. Measured volumes of buffer and metoprolol stock solution were placed in the vessel, and the change in the concentration of oxygen was monitored while the mixture was continuously stirred. The oxygen content of the solutions became constant after about 50 s, when a known volume of H_2O_2 stock solution was added. After about 50 s the concentration of oxygen reached a constant value. Now, the catalyzed reaction was started by a quick adding of 2 mL of the enzyme stock solutions by means of syringe. A typical oxygen concentration versus time curve obtained by monitoring the reaction with a Clark sensor is presented in Fig. 1. The rate has been measured after the introduction of the enzyme solution into the mixture as the slope of the oxygen concentration increase with time. Only the starting period, within 25 – 30 % of reaction, with a linear dependence has been considered. The degree of H_2O_2 transformation was calculated from the experimental measured oxygen concentration as

$$X = \frac{2[O_2]_t - [O_2]_0}{[H_2O_2]_0} \quad (2)$$

where $[O_2]_t$ is the actual concentration of O_2, and $[O_2]_0$ is the constant value of $[O_2]$ obtained after the addition and consumption of the whole amount of H_2O_2.

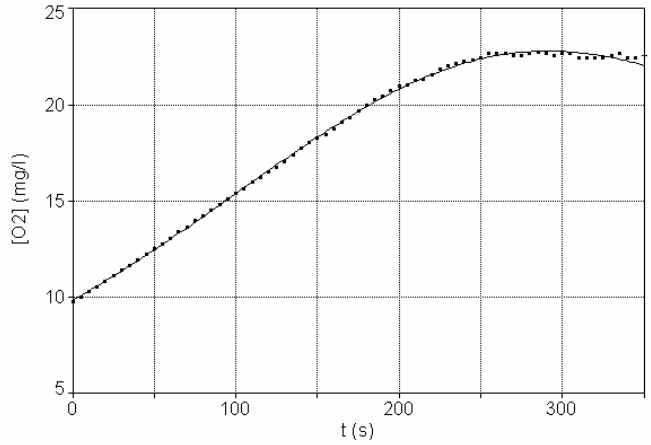


Fig. 1. A typical curve for O_2 evolution, recorded with the Clark sensor in the reaction mixture pH=7 at 20^0C

RESULTS AND DISCUSSION

A large amount of information is available in the literature concerning the kinetics and the mechanism [4, 26, 27, 28] of the reaction in the absence of inhibitor. Although the catalase-H_2O_2 complex formed in the first step of the reaction interacts with another hydrogen peroxide molecule [29] and some exchange of the valence state

of iron ion takes place, we obtained a Michaelis behavior of the over-all reaction (1) with the initial rates.

The initial reaction rates were determined from the slopes of the early part of the O_2 evolution curves, after catalase addition. According to the well-known Michaelis-Menten equation [30]

$$r_0 = \frac{k[E_0][S]}{K_M + [S]} = \frac{r_{max}[S]}{K_M + [S]} \quad (3)$$

where $[E]_0$ and [S] stand for the initial concentration of the enzyme and for the substrate concentration respectively, K_M and r_{max} are the Michaelis-Menten parameters, and k is the rate constant of breakdown of the enzyme substrate complex to the product. This equation can be brought into the double-reciprocal form:

$$\frac{1}{r_0} = \frac{1}{r_{max}} + \frac{K_M}{r_{max}} \cdot \frac{1}{[S]} \quad (4)$$

and used to obtain the Lineweaver-Burk plots [31]. The value of the Michaelis-Menten constant K_M= 4.55x10^{-4} mol/L and the maximum velocity r_{max}=5.11x10^{-2} mol L^{-1} s^{-1}, obtained by us, are comparable to those mentioned in literature[32]. And dose to those determined in our previous study. [12]

The influence of metoprolol on the enzyme-catalyzed decomposition of hydrogen peroxide .Several kinetic runs performed in the presence of different concentrations of metoprolol proved the inhibitory effect of this compound. When the concentration of H_2O_2 was varied at several chosen concentrations of metoprolol, Lineweaver-Burk plots for the inhibited reaction

$$\frac{1}{r_{o(I)}} = \frac{1}{r_{max(I)}} + \frac{K_{M(I)}}{r_{max(I)}} \cdot \frac{1}{[S]} \quad (5)$$

resulted in a family of straight lines (Fig. 2), which have a common intersection point on the left side of the ordinate and below the abscissa.

Table 1

Equations of Lineweaver-Burk plots for different concentration of metoprolol

$[Metoprolol]_0 x10^7$ (mol/L)	Intercept (L.s/mol)	Slope (s)
0	19.538	0.0089
1	23.147	0.0095
2	27.150	0.0099
3	37.120	0.012

As the slopes of Lineweaver- Burk plots depend on the inhibitor concentration, a systematic study could lead to a kinetic method of determination of this inhibiting agent concentration. The Michaelis-Menten parameters in the presence of the inhibitor were determined from the parameters of the lines in figure 2. Their values are given by the equations (6):

$$r_{max(I)} = r_{max} \frac{1 + \beta \frac{[I]}{K'_I}}{1 + \frac{[I]}{K'_I}} \quad \text{and} \quad K_{M(I)} = K_M \left(\frac{1 + \frac{[I]}{K_I}}{1 + \frac{[I]}{K'_I}} \right) \tag{6}$$

where: K'_I and K_I are the dissociation constants of the enzyme – substrate - inhibitor complex, ESI, and the enzyme. inhibitor complex, EI. The constant β is equal to zero for full inhibition, while for partial inhibition $0< \beta \leq 1$. Lineweaver- Burk plots of Fig. 2 correspond to the inhibition pattern of either full or partial mixed inhibitors [33].

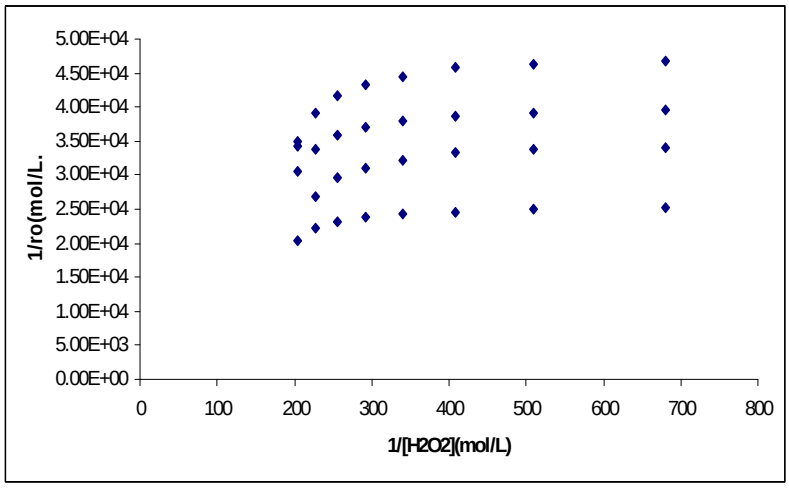


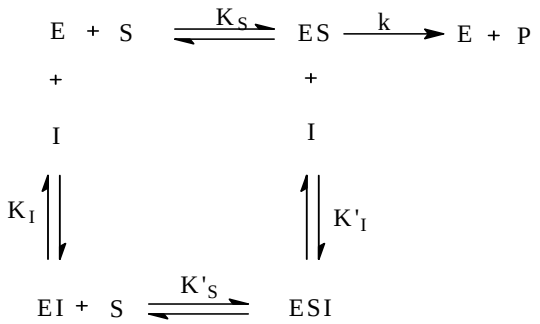
Fig.2. Lineweaver Burk plots obtained for different concentration of metoprolol

In order to distinguish between full and partial inhibition, the slopes and intercepts from the primary Lineweaver- Burk plots were re-plotted against the corresponding inhibitor concentration. They are presented in figure 3. Both plots gave straight lines. This behavior is considered as typical for full inhibition [24]. Considering the possibility of a partial inhibition, a non-linear fit of the intercept $1/r_{max(I)}=f([I]$ and the slope $K_{M(I)}/r_{max(I)}=f$ [I] gave negative values for β. Therefore it is very likely that metoprolol acts as a reversible full mixed inhibitor according to the reaction scheme 1

The plot (Fig.3) of slopes and intercepts respectively, against the inhibitor concentration were used to determine the dissociation constants mentioned in the scheme above, $K_I=8.86\text{x}10^{-7}$ M for EI complex and $K'_I=3.04\text{x}10^{-7}$ M for ESI complex . The constant K_I was obtained from the slope of the $K_{M(I)}/r_{max(I)}$ versus [Metoprolol] graph:

$$K_I = \frac{K_M}{r_{max} \cdot slope} \tag{7}$$

while the K'_I constant was determined from the slope of the $1/r_{max(I)}$ versus [metoprolol] graph:



Scheme 1. Mixed inhibition mechanism for metoprolol, where E stands for catalase, S for hydrogen peroxide and I for metoprolol.

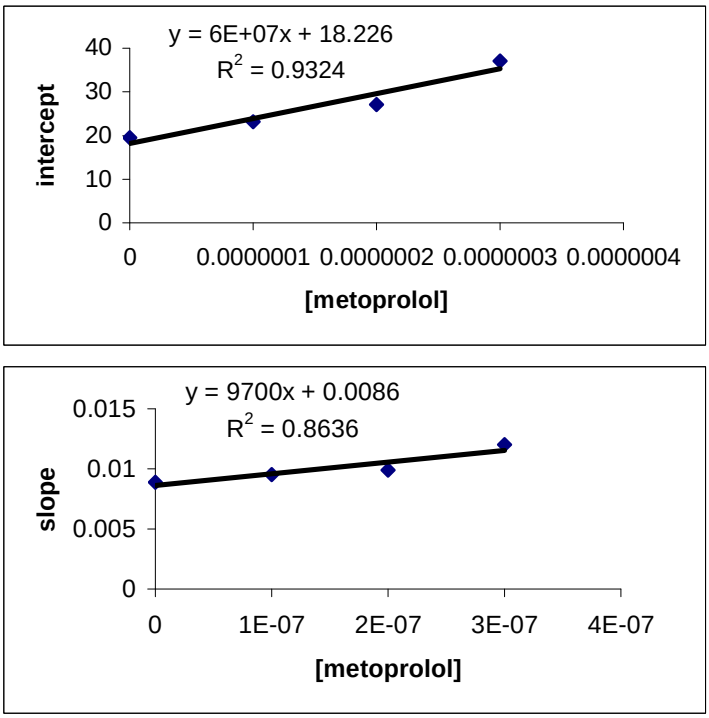


Fig 3. The plot of intercepts and slopes versus the inhibitor concentration

$$K'_I = \frac{1}{r_{max \cdot slope}} \quad (8)$$

Table 2

Mean values of $r_{max(I)}$ and Michaelis constant in the presence of the inhibitor $K_{M(I)}$

$[Metoprolol]_0 \times 10^7$(mol/l)	$r_{max(I)} \times 10^2$(mol/Ls)	$K_{M(I)} \times 10^4$(mol/L)
0.00	5.11	4.55
1.00	4.32	4.10
2.00	3.68	3.64
3.00	2.69	3.23

Kinetic method for the determination of metoprolol

We tried to exploit the inhibitory effect of metoprolol, upon the catalytic reaction of catalase, for the determination of this compound by means a kinetic method.The method consist in the monitoring of the oxygen evolution by means of Clark sensor The results were employed to obtain a calibration graph of initial rate against metoprolol concentration at fixed concentration of H_2O_2 and catalase. The graph exhibit a non linear aspect as described by the equation (9) and presented in figure 4.

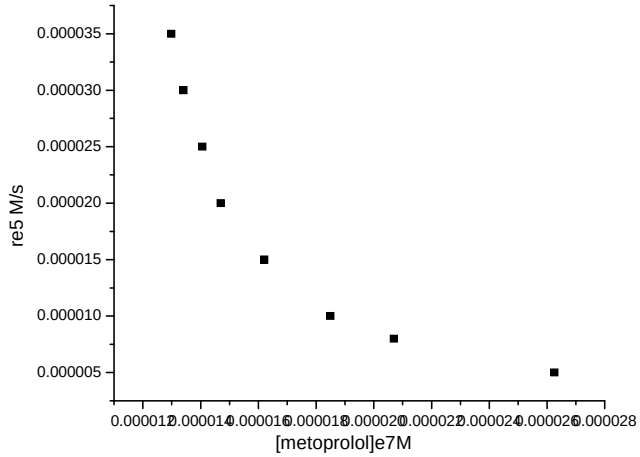


Fig 4. Calibration graph for metoprolol

$$r_{(I)} = \frac{r_{max} \cdot [S]}{\frac{K_M}{K_I} \cdot [(K_I + [I]) + [S \cdot (K'_I) + [I]]]} \quad (9)$$

Its linear form is presented in figure 5.

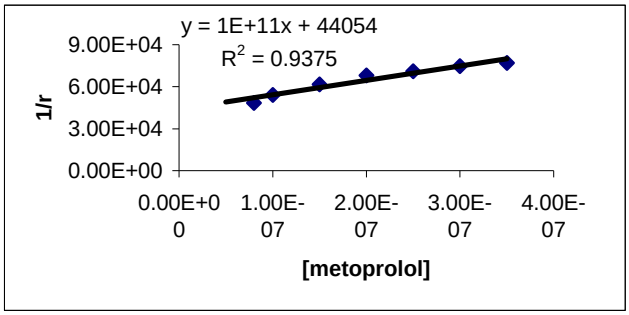


Fig 5. Linear calibration for metoprolol

It is obvious that heavy metals ions (Cu^{2+}, Fe^{3+}, Zn^{2+}, Ni^{2+}) will influence the reaction rate, because they have a catalytic effect on the decompositions of H_2O_2, but according to the literature there are many organic compounds (especially aromatic compounds) that may interfere too, because they act as inhibitors of catalase, including: naphthalene, 2-naphthol, 2- naphthalene-sulfonic acid, benzene, phenol, benzenesulfonic acid, menthol, inositol, biotin, procaine, sulfanilamide, pyridoxine, folic acid, aminopterin,and riboflavine [34]

Further sturdy will elucidate the potential interference on this kinetic method.

CONCLUSIONS

The catalase –catalyzed decomposition of hydrogen peroxide in phosphate buffer was studied in the presence of metoprolol. The metoprolol acts like a fully mixed inhibitor, as observed from Lineweaver-Burk plots.

Michaelis Menten parameters were determined for the decomposition of H_2O_2 in the present of catalase yielding the values of $K_M = 4.55 \times 10^{-4}$ mol/L and

$$r_{max} = 5.11 \times 10^{-2} mol/L^{-1}s^{-1}$$

Linear dependence of the slope and respectively intercept of Lineweaver-Burk linearisations from metoprolol concentration indicates a fully mixed inhibition mechanism.

A kinetic method was suggested for the determination of metoprolol according to its inhibitory effect.

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