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A kinetic determination of lysine in pharmaceutical sample

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Abstract: A kinetic method for the determination of micro quantities of the amino acid lysine (Lys) is described in this paper. The catalytic activity of cobalt in the reaction of the oxidation of purpurin (1,2,4-trihydroxyanthraquinone, PP) by hydrogen peroxide in alkaline buffer solution decreases in the presence of micro quantities of lysine, because of the formation of a complex.[1,2] The experimental conditions for the successful determination of lysine were optimized. The relative error ranges from 7.7 to 1.9 % in the concentration range from 0.118 to 23.520 μg cm^{-3}. The detection limit is 0.023 μg cm^{-3}. Kinetic equations are proposed for the investigated process. The effects of certain foreign ions and amino acids on the reaction rate were determined in order to assess the selectivity of the method. The method was applied to the determination of lysine in a pharmaceutical sample Riborn-P (Medecon–Belgrade).

Keywords: kinetic method, lysine, purpurin oxidation, pharmaceutical sample.

INTRODUCTION

Lysine is an essential amino acid which has been recognized since 1889. Lysine is involved in several metabolic pathways[3] and is often the first limiting amino acid in pig and poultry diets.[4] The need for lysine is increased in women during pregnancy. Metabolic disorders of lysine catabolism can lead to periodical and permanent hyperlysanamia, where in the latter case, some but not all patients are mentally retarded.[5,6]

Bearing in mind the need for essential amino acids, as well as disorders in the synthesis of amino acids that can cause a large number of illnesses, it is necessary to take good care of the quality of food and pharmaceutical preparations (regarding the amounts of amino acids) by means of which they are taken into the human body.

There are many detailed kinetic studies concerning the determination of ultramicro quantities of inorganic ions.[7] On the contrary, there is a smaller number of reports dealing with kinetic methods for the determination of ultramicro quantities of organic compounds, essential to amino acids. Micro amounts of lysine have been determined by chemiluminescence. The determination is based on the inhibition of the enhancement effect of Cu(II) on the luminol + H_2O_2 chemiluminescence. The minimum detection of lysine is

several ng per injection.[8] In this paper a kinetic method which was applied to determine lysine with a sensitivity of 0.118 μg cm^{-3} is described. The oxidation of purpurin with hydrogen peroxide in the presence of Co(II) as a catalyst in a carbonate buffer solution gives a colorless product. It was observed that a small amount of lysine inhibits this reaction. The rate of the reaction is inversely proportional to the concentration of this amino acid. This fact was used as the basis of a kinetic method for the determination of micro quantities of lysine.

EXPERIMENTAL

Apparatus

The reaction rate of the oxidation of purpurin by hydrogen peroxide was followed photometrically. The absorbance of the solution was measured at a wavelength of 540 nm, which corresponds to the maximum absorbtion of purpurin in carbonate buffer at pH 10.25. The readings were obtained using a Perkin-Elmer Lamda 15 UV/VIS spectrophotometer connected to a thermocirculating bath. A Radiometer PHM 29b pH meter was used to measure the pH values of the solution. Sigma buffers, pH 7±0.01 and pH 4±0.01 were used for the calibration the pH meter. The solutions were thermostated at 22 ± 0.1 ºC before the beginning of the reactions.

Reagents

A solution of purpurin (1×10^{-3} mol dm^{-3}) was prepared by dissolving the required amount in 5 cm^3 0.1 mol dm^{-3} NaOH, followed by dilution with water to a total volume 100 cm^3. The carbonate buffer [HCO_3^-] / [CO_3^{2-}] (1 mol dm^{-3}) was obtained by mixing $NaHCO_3$ and Na_2CO_3 in the appropriate ratio. A hydrogen peroxide solution (4×10^{-2} mol dm^{-3}) was prepared from a 35 % commercial reagent. A stock Co(II) solution (1.7×10^{-2} mol dm^{-3}) was prepared by dissolving $CoCl_2\times6H_2O$ in water. The working solution (1.7×10^{-6} mol dm^{-3}) was obtained by diluting the stock Co(II) solution with water. The amino acid solution (1×10^{-3} mol dm^{-3}) was prepared by dissolving lysine in water. Analytical grade chemicals ("Merck") and deionised water were used for the preparation of all solutions. All the working solutions were prepared just before use.

All the glassware used was washed with aqueous HCl (1:1) and then thoroughly rinsed with running, distilled and finally with deionised water.

Procedure

The reaction was carried out in the following way : In the reaction – mixture vessel with four compartments, a solution of purpurin and buffer was placed in one compartment, hydrogen peroxide in the second, cobalt(II) in the third, and lysine and water (total volume 10 cm^3) in the fourth compartment.

The vessel was thermostated at 22 ± 0.1 ºC and the reaction was initiated by vigorous mixing. The reaction solution was put into a cell and the absorbance at 540 nm was measured photometrically every 30 s over the next 5 min.

RESULTS AND DISCUSSION

Kinetic studies

A differential variant of the tangent method was used for processing the kinetic data, because of the existence of a linear correlation between the absorbance and time during the initial 5 min period. The reaction rate was followed by the change in the values of the tangent of the angle (tan α) of the slope in the linear part of the kinetic curve to the abcissa in the coordinates A–t, because of $\tan\alpha = \mathrm{d}A/\mathrm{d}t$.

In order to determine the lowest possible determinable concentration of lysine, the conditions needed to be optimized. Therefore, the dependencies of the rates of both the catalytic and the inhibited reaction on the concentration of each of the reactants were determined.

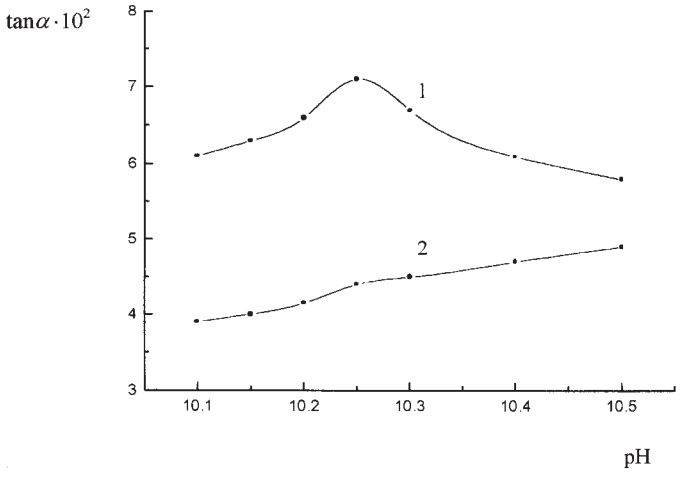


Fig. 1. Depencence of the rate of the catalysed (1) and inhibited (2) reaction on the pH. Initial concentrations: $c_{pp} = 1 \times 10^{-5}$ mol dm^{-3}, $c_{buff} = 0.2$ mol dm^{-3}, $c_{H_2O_2} = 4 \times 10^{-3}$ mol dm^{-3}, $c_{Co}^{2+} = 3.4 \times 10^{-7}$ mol dm^{-3}, $c_{Lys} = 3 \times 10^{-4}$ mol dm^{-3}, $t = 22 \pm 0.1$ °C.

Figure 1 shows the influence of pH on the rate of both reactions. It can be seen that the greatest difference between the reaction rates occurs at pH 10.25. For further work, this pH value was selected. From Fig. 1, it can be seen that the dependencies of the rates of the inhibited and the catalysed reaction on pH are not linear. Hence, the logarithm of tan α for both reactions was found. From the linear correlations between the logarithm of tan α and pH, the orders of both reactions were determined. For the catalysed reaction, it was 0.4 and for the inhibited 0.3 in the pH interval from 10.10 – 10.25.

The dependence of tan α on the buffer concentration is presented in Fig. 2. It shows that the difference in the rates of the inhibited and catalysed reactions increases with increasing buffer concentration in the range from 0.10–0.13 mol dm^{-3} and that both reac-

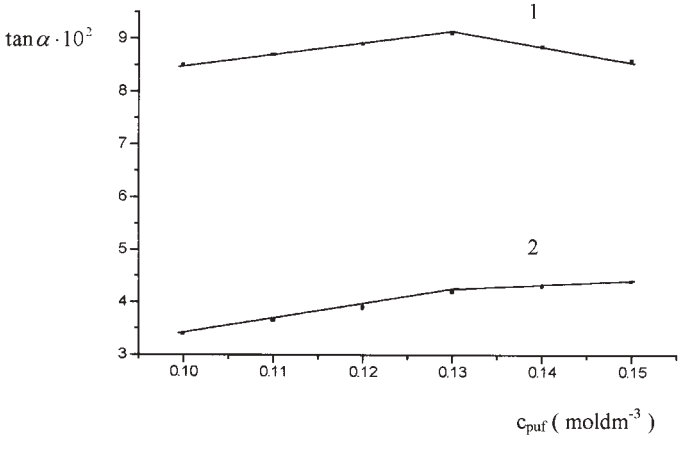


Fig. 2. Dependence of the rate of the catalysed (1) and inhibited (2) reaction on the buffer concentration. Initial concentrations: pH 10.25, $c_{pp} = 1 \times 10^{-5}$ mol dm^{-3}, $c_{H_2O_2} = 4 \times 10^{-3}$ mol dm^{-3}, $c_{Co}^{2+} = 3.4 \times 10^{-7}$ mol dm^{-3}, $c_{Lys} = 3 \times 10^{-4}$ mol dm^{-3}, $t = 22 \pm 0.1$ °C.

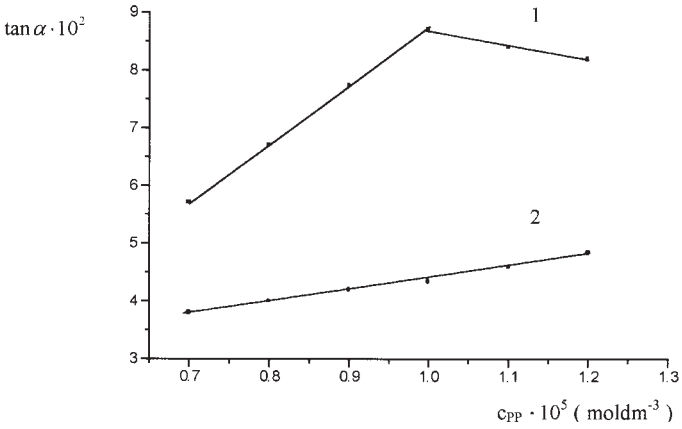


Fig. 3. Dependence of the rate of the catalysed (1) and inhibited (2) reaction on the purpurin concentration. Initial concentrations: pH 10.25, c_{buff} = 0.13 mol dm^{-3}, $c_{H_2O_2} = 4 \times 10^{-3}$ mol dm^{-3}, $c_{Co^{2+}} = 3.4 \times 10^{-7}$ mol dm^{-3}, $c_{Lys} = 3 \times 10^{-4}$ mol dm^{-3}, $t = 22 \pm 0.1$ °C.

tions are first order with respect to the buffer concentration in the mentioned concentration interval. At concentrations higher than 0.13 mol dm^{-3}, the catalysed reaction is negative and the inhibited is positive first order. For further work, a buffer concentration of 0.13 mol dm^{-3} was selected.

The correlation between tan α and the purpurin concentration is shown in Fig. 3. The catalysed reaction is first order with respect to the purpurin concentration in the concentration range from 0.7 – 1.0×10^{-5} mol dm^{-3} and negative first order at concentrations higher than 1.0×10^{-5} mol dm^{-3}. The inhibited reaction is positive first order with respect to the purpurin concentration. For further work, a purpurin concentration of 1.0×10^{-5} mol dm^{-3} was selected, because at this concentration the difference between the reaction rates is the greatest.

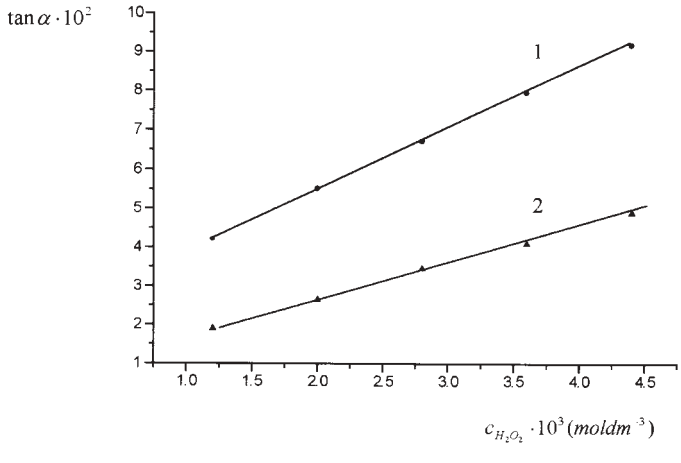
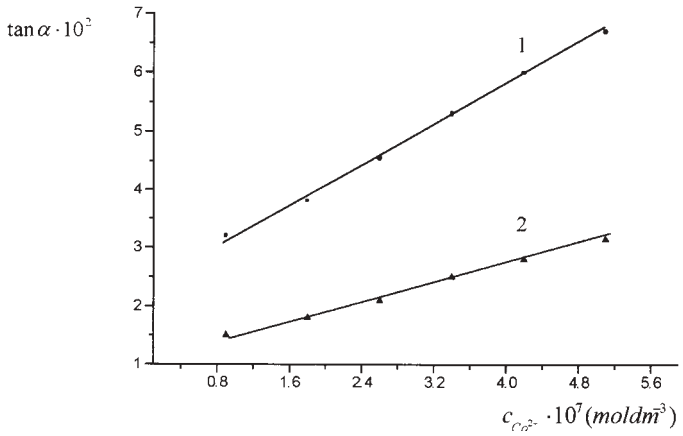


Fig. 4. Dependence of the rate of the catalysed (1) and inhibited (2) reaction on the H_2O_2 concentration. Initial concentrations: pH 10.25, $c_{pp} = 1 \times 10^{-5}$ mol dm^{-3}, $c_{buff} = 0.13$ mol dm^{-3}, $c_{Co^{2+}} = 3.4 \times 10^{-7}$ mol dm^{-3}, $c_{Lys} = 3 \times 10^{-4}$ mol dm^{-3}, $t = 22 \pm 0.1$ °C.

The dependence of the reaction rates on the H_2O_2 concentration is presented in Fig. 4. Both reactions are first order with respect to the H_2O_2 concentration. For further work, an H_2O_2 concentration of 2×10^{-3} mol dm^{-3} was selected, because at higher concentrations the rate of both reaction becomes too fast and so not suitable for kinetic determinations.

The correlations between tan α and the Co(II) concentration are shown in Fig. 5. It can be seen that both reactions are first order with respect to the Co(II) concentration. For further work a Co(II) concentration of 4.2×10^{-7} mol dm^{-3} was selected, since at higher concentrations the linear part of the kinetic A–t curve is rather short.



Fi.g 5. Dependence of the rate of the catalysed (1) and inhibited (2) reaction on the Co(II) concentration. Initial concentrations: pH 10.25, $c_{pp} = 1 \times 10^{-5}$ mol dm^{-3}, $c_{buff} = 0.13$ mol dm^{-3}, $c_{H2O2} = 2 \times 10^{-3}$ mol dm^{-3}, $c_{Lys} = 3 \times 10^{-4}$ mol dm^{-3}, $t = 22 \pm 0.1$ °C.

Under the optimal reaction conditions ($c_{pp} = 1\times10^{-5}$ mol dm^{-3}, $c_{buffer} = 0.13$ mol dm^{-3}, $c_{H_2O_2} = 2\times10^{-3}$ mol dm^{-3}, $c_{Co(II)} = 4.2\times10^{-7}$ mol dm^{-3}, pH 10.25, $t = 22 \pm 0.1$ °C) the lysine concentration was varied from 0.118 to 23.520 μg cm^{-3} (0.8×10^{-6} – 1.6×10^{-4} mol dm^{-3}).

The equations of the dependence of tan α on the lysine concentration and the recovery (r) for the determination of lysine in the mentioned interval were calculated.

In the concentration range from 0.118–1.470 μg cm^{-3} (Fig. 6):

$$y = -0.00431 c_{Lys} + 0.04086; \qquad r = 99.75\ \%$$

In the concentation range from 1.470 – 23.520 μg cm^{-3} (Fig. 7):

$$y = -0.00031 c_{Lys} + 0.0350; \qquad r = 99.86\ \%$$

Figs. 6 and 7 present calibration curves at 22 °C which can be used for the determination of the lysine concentration in the given concentration ranges.

The following kinetic equations for the investigated process were deduced on the basis of the graphic correlations obtained.

For the catalysed reaction:

$$-\frac{dc_{pp}}{dt} = k_1\, c_{pp}\, c_{buff}\, c_{H_2O_2}\, c^{0.4}_{H_3O^+}\, c_{Co^{2+}} \qquad (1)$$

For the inhibited reaction:

$$-\frac{dc_{pp}}{dt} = k_2\ c_{pp}\ c_{buff}\ c_{H_2O_2}\ c_{H_3O^+}^{0.3}\ c_{Co^{2+}}\ c_{Lys}^{-1} \qquad (2)$$

where: k_1 – constant proportional to the rate constant of the catalysed reaction and k_2 – constant proportional to the rate constant of the inhibited reaction.

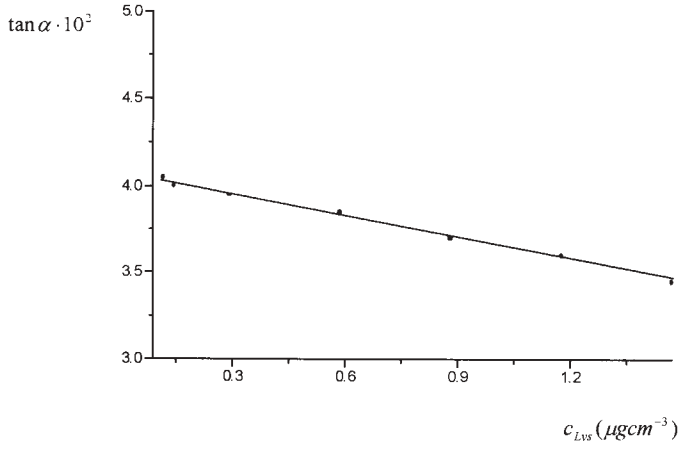


Fig. 6. Dependence of the rate of the inhibited reaction on the lysine concentration-calibration curve at 295 K (22 ºC). Initial concentrations: pH 10.25, c_{pp} = 1 × 10^{-5} mol dm^{-3}, c_{buff} = 0.13 mol dm^{-3}, c_{H2O2} = 2 × 10^{-3} mol dm^{-3}, c_{Co}^{2+} = 4.2 × 10^{-7} mol dm^{-3}.

The equations are valid for the following concentrations: PP 0.7×10^{-5} – 1.0×10^{-5} mol dm^{-3} and 0.7×10^{-5} – 1.3×10^{-5} mol dm^{-3} (Eqs. (l) and (2)), buffer 0.10 – 0.13 mol dm^{-3} and 0.10 – 0.15 mol dm^{-3} (Eqs. (l) and (2)), H_2O_2 1.2×10^{-3} – 4.4×10^{-3} mol dm^{-3} (Eqs. (l) and (2), pH 10.10 – 10.25 (Eqs. (l) and (2)), Co(II) 0.9 – 5.1×10^{-7} mol dm^{-3} (Eqs. (l) and (2)), lysine 0.118 – 23.520 μg cm^{-3} (0.8×10^{-6} – 1.6×10^{-4} mol dm^{-3}) (Eq. (2)).

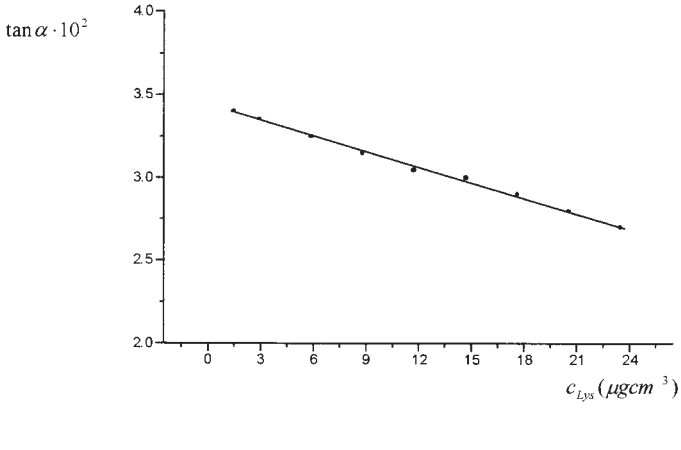


Fig. 7. Dependence of the rate of the inhibited reaction on the lysine concentration–calibration curve at 295 K (22 ºC). Initial concentrations: pH 10.25, c_{pp} = 1 × 10^{-5} mol dm^{-3}, c_{buff} = 0.13 mol dm^{-3}, c_{H2O2} = 2 × 10^{-3} mol dm^{-3}, c_{Co}^{2+} = 4.2 × 10^{-7} mol dm^{-3}.

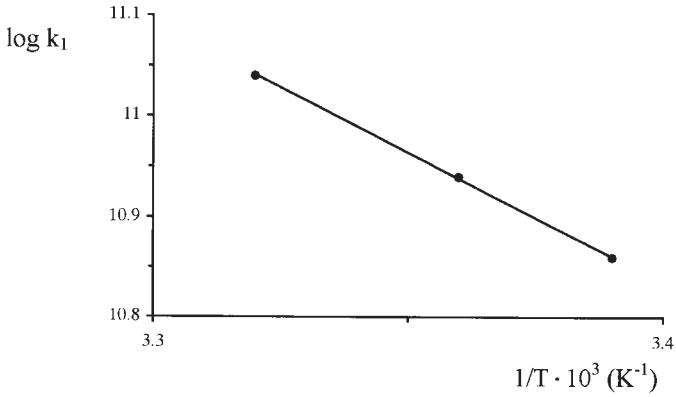


Fig. 8. Dependence of the logarithm of the rate constant of the catalysed reaction on the reciprocal of the absolute temperature.

On the basis of these equations, the rate constants for the inhibited and catalysed reactions were calculated (Table I).

TABLE I. Rate constants for the catalysed (k_1) and inhibited (k_2) reactions at three temperatures

$k_1 \times 10^{-10}$/(mol dm^{-3})$^{1-n}$s^{-1}	$k_2 \times 10^{-10}$/(mol dm^{-3})$^{1-n}$s^{-1}	T/K
7.30	5.54	295
9.12	5.65	298
10.08	5.71	301

n – order of the reaction

A linear relationship between the logarithm of the rate constant and the reciprocal of the absolute temperature was found for both the catalysed (Fig. 8) and the inhibited (Fig. 9) reaction. The activation energies were found to be 49.76 kJ mol^{-1} for the catalysed reaction and 59.33 kJ mol^{-1} for the inhibited reaction.

The minimum concentration of lysine which can be determined by this method may be calculated by the method given by Perez-Bendito and Silva.[9,10] The detection limit was found to be 0.023 μg cm^{-3}, which is five time less than the real concentration which was 0.118 μg cm^{-3}.

The accuracy and precision of the measurements are presented in Table II.[11] The relative error ranges from 7.7–1.9 % for lysine concentrations ranging from 0.118–23.520 μg cm^{-3}.

To assess the selectivity of the method, the influence of several foreign ions and amino acids on the rate of the inhibited reaction was studied at a constant lysine concentration of 11.760 μg cm^{-3} (Table III). It can be seen that Ni^{2+}, Fe^{3+}, Asn, Ala, Gly, Arg, His in a 0.1:1 ratio and Sn^{2+}, Cd^{2+}, Mg^{2+}, Al^{3+}, SO_4^{2-}, $C_2O_4^{2-}$, NO_3^-, F^- in a 1:1 ratio with lysine interfered with the reaction. Cu^{2+} and Mn^{2+} catalysed this reaction. The other investigated ions had practically no influence on the determination of lysine by this method. Since organic molecules are concerned, the selecitivity was expected.

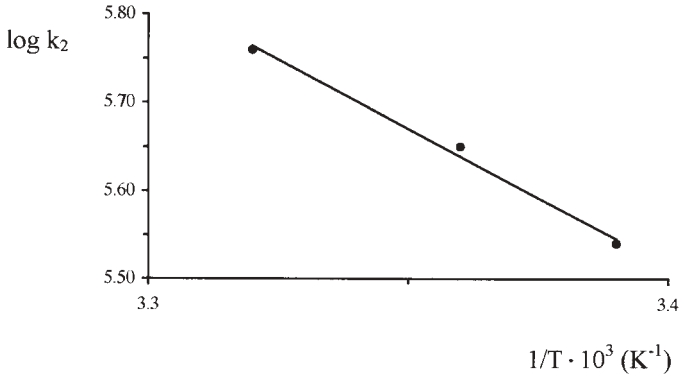


Fig. 9. Dependence of the logarithm of the rate constant of the inhibited reaction on the reciprocal of the absolute temperature.

TABLE II. Accuracy and precision of the determination of lysine

Taken ($\bar{x}$)/μg cm^{-3}	Found ($\bar{x}$)/μg cm^{-3}	n	$S_{\bar{x}}$/μg cm^{-3}	G/%	$(x - \mu)/\mu \cdot 100$
0.147	0.156	5	0.04	7.7	–6.7
0.588	0.608	5	0.14	6.3	–3.4
1.470	1.499	5	0.34	5.9	2.0
11.760	11.666	5	1.34	3.2	–0.8
23.520	23.331	5	1.67	1.9	–0.8

$\bar{x}$ – Mean value, n – number of measurements, S_x – standard deviation of the mean value, G – relative error, $(x - \mu) / \mu \cdot 100$ – accuracy of the method, μ – real value

TABLE III. Effect of foreign ions and amino acids on the determination of lysine

Tolerance level c_{ion}/c_{Lys}	Ion added
10	CH_3COO^-; HPO_4^-; PO_4^{3-}; Citrate
1	HCO_3^-; SO_4^{2-}; $C_2O_4^{2-}$; F^-; NO_3^-; Al^{3+}; NH^{4+}; Sn^{2+}; Cd^{2+}; Mg^{2+}; Phe; Tyr; Met; Trp; Ser
0.1	Fe^{3+}; Ni^{2+}; Asn; Ala; Gly; Arg; His
catalysed	Cu^{2+}; Mn^{2+}

Determination of lysine in a pharmaceutical preparation

The developed method was directly applied to the determination of lysine in the pharmaceutical preparation: Riborn-P. Since Riborn-P is a protein imuno-complex and therefore has small quantities of amino acids together with large quantities of other substances such as vitamins and proteins in the form of gluconates which, considering their given proportion in the preparation, affect the determination of lysine, it was necessary to combine several methods for the isolation of lysine and its determination.

Firstly, the amino acids were seprated from the mixture using 80 % ethanol and the proteins were precipitated with chloroform; next, the basic amino acids (histidine, lysine, arginine) were separated; finally, lysine was separated from arginine and histidine.[12] To 0.5 g of the sample in an Erlenmeyer flask was added 4 cm^3 of deionized water together with 16 cm^3 of abso-

lute ethanol. The contents of the flask were shaken for half an hour. Then the mixture in the flask was transferred to a 100 cm^3 separation funnel and 60 cm^3 of chloroform added and all was well shaken. The upper aqueous layer containing the amino acids was separated and used for the isolation of the basic amino acids. Kossel and Kutscher formulated a quantitative method for the separation of basic amino acids : arginine, histidine and lysine.[12] A warm concentrated solution of Ag_2SO_4 in a small surplus was added to the aqueous solution of amino acids. Arginine and histidine were precipitated out of the solution by the addition of $Ba(OH)_2$, while lysine remained in the solution. The silver ions were removed by adding 0.1 mol dm^{-3} $Na_2S_2O_3$ solution which binds silver ions into a stable complex. Employing multiple chloroform extractions, the thiosulphate silver complex was extracted from the aqueous solution. Following the appropriate preparation of the samples and their dilution for the analysis, the quantity of lysine in the samples was determined using the above established calibration curves.

The accuracy and precision of the determintion of lysine in the pharmaceutical sample by the proposed kinetic method were calculated (Table IV).

TABLE IV. Accuracy and pecision of the determination of lysine using the obtained calibration curves.

Sample	Taken ($\bar{x}$)/μg cm^{-3}	Found ($\bar{x}$)/μg cm^{-3}	n	$S_{\bar{x}}$/μg cm^{-3}	G/(%)	$(x - \mu) / \mu \cdot 10$
Riborn-P	11.760	10.334	5	6.58	6.4	–12.1

Regardless of the inevitable loss of lysine, which is understandable considering the fact that several stages were necessary for the separation of this amino acid, the above discussed kinetic method can be applied to determine lysine in the given pharmaceutical sample.

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ИЗВОД

КИНЕТИЧКО ОДРЕЂИВАЊЕ ЛИЗИНА У ФАРМАЦЕУТСКОМ ПРЕПАРАТУ

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Предложена је нова кинетичка метода за одређивање микрограмских количина аминокиселине лизина (Lys), која се заснива на његовом инхибиторном ефекту у реакцији оксидације пурпурина (1,2,4-трихидроксиантрахинона, PP) водоник-пероксидом у базној средини карбонатног пуфера у присуству Co(II) јона као катализатора. Одређени су оптимални услови одигравања реакције. Релативна грешка методе се креће од 7,7–1,9 % за интервал концентрације лизина од 0,118–23,520 μg cm^{-3}. Дате су кинетичке једначине за предложене процесе. Испитан је утицај већег броја страних јона и аминокиселина на брзину реакције. Метода је примењена за одређивање лизина у фармацеутском препарату Riborn-P (Medecon-Beograd).

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