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Inhibitory kinetic spectrophotometric method for the quantitative estimation of D-penicillamine at micro levels

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ABSTRACT

Biologically active thiol, D-penicillamine (D-PA), based on the hard soft acid base (HSAB) principle, binds strongly with Hg(II) ions leading to the inhibition of the rate of Hg(II) catalyzed substitution of cyanide in hexacyanoruthenate(II) i.e. $[Ru(CN)_6]^{4-}$ by nitroso-R-salt (NRS). This formed the basis for the development of a novel kinetic spectrophotometric method for the quantitative determination of D-PA at micro levels. The reaction was followed spectrophotometrically at 525 nm (λ_{max} of $[Ru(CN)_5NRS]^{3-}$ complex) under optimized reaction conditions at 8.75×10^{-5} M $[Ru(CN)_6^{4-}]$, 3.50×10^{-4} M [NRS], pH 7.00 ± 0.02, ionic strength (μ) 0.1 M (KCl) and temperature 45.0 ± 0.1 °C. The modified mechanistic scheme is proposed to understand the inhibition caused by D-PA on Hg(II) catalyzed substitution of cyanide by NRS in $[Ru(CN)_6]^{4-}$. The detection limit of the proposed method was found to be 2.5×10^{-7} M. The method has successfully been applied for the determination of D-PA in real samples with quantitative results.

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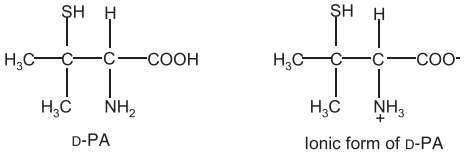
1. Introduction

The sulfur-containing compounds are involved in several important biochemical processes, including but not limited to redox regulation and drug conjugation/detoxification [1]. Thus, there has been continuous development of analytical methodology using different techniques for the detection and determination of sulfur-containing compounds in different samples [2–5]. Penicillamine is a medication of the chelator class and sold under the trade names of Cuprimine and Depen [6]. It exists in two enantiomeric forms; D-penicillamine and L-penicillamine. Fundamentally, Cuprimine and Depen is D-penicillamine (D-PA) because L-penicillamine (L-PA) is quite toxic [4,6]. The World Health Organization has listed D-PA as an essential medicine, the most important medication needed in a basic health system [7]. D-PA is a sulfur containing amino acid which belongs to aminothiol family where a hydrogen atom in the β-carbon of cysteine is replaced by the methyl group [4,8] and has found many therapeutic applications [8–10]. Therefore, D-PA, whose structure is shown below, is among the most often used thiols as therapeutic substance [7,11].



Structure of D-Penicillamine

D-PA i.e. 3-mercapto-D-valine is a characteristic acid degradation product of β-lactam antibiotics [12]. It is used for the treatment of rheumatoid arthritis [13], cystinuria and in heavy metal poisoning [6,13,14]. Due to its ability of chelation, it is also used for the elimination of copper in the treatment of hepatolenticular degeneration i.e. Wilson's disease [13,14] and has been investigated as an antiangiogenic agent [14–16]. Based on its wide applications in the treatment of a number of diseases and disorders in the human body, it has been categorized as a biologically active thiol [6,17]. One perceived action of thiols in vivo is that they act as anti-oxidants [18] which protect cells and tissues by actively scavenging oxygen species [19,20]. Thus, the development of an analytical method for the quantitative determination of D-PA is of great importance and may receive the attention of pharmaceutical chemists.

There are a number of methods that have been reported for the determination of D-PA in pharmaceutical preparations, analytical and biological samples. These methods include colorimetry [21–29], fluorimetry [12,30], chromatography [8,17,31–38], flow injection analysis

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[39–41], electrophoresis [42–44], potentiometry [11,45,46], voltammetry [10,47–53], NMR-spectrometry [54,55] and spectrophotometry [9,21,23,24,26,27,56] while very few kinetic methods have been reported using different detection techniques [4,11,57]. The analytical methods developed for the detection and determination of D-PA before 1981 have been reviewed by Chiu and Grady [58] and Kucharczyk and Shariman [59]. Some of the main drawbacks in many of the reported methods are high initial investment, heavy instrumentation, high cost of per sample analysis, etc.

The kinetics and mechanism of Hg(II) catalyzed substitution of cyanide in $[Ru(CN)_6]^{4-}$ by NRS have been recently reported by us [60]. This reaction has successfully been utilized for the development of a novel method for the determination of Hg(II) based on its catalytic effect [61] and sodium thiosulphate (STH), thioglycolic acid (TGA) based on their inhibitory effect on this indicator reaction system [62]. Hg(II) is a soft acid and has very good affinity towards sulfur containing ligands [62,63]. Based on the hard soft acid base (HSAB) principle, which has widely been used in chemistry for explaining the stability of complexes [64], it has been observed that D-PA strongly inhibits the rate of Hg(II) catalyzed substitution of cyanide in $[Ru(CN)_6]^{4-}$ by nitroso-R-salt (NRS) [60]. To the best of our knowledge, no analytical method has been reported for the determination of D-PA, in pure or pharmaceutical preparations, based on ligand substitution reaction involving substitution of cyanide in $[Ru(CN)_6]^{4-}$ by NRS except the one recently reported by us which is based on the slow uncatalyzed ligand substitution reaction between $[Fe(CN)_5(H_2O)]^{3-}$ and D-PA [4]. We have been interested in developing methods for various analytes of environmental, biological and medicinal interest [4,5,61–63,65–67]. Keeping the above explained backgrounds in mind, in the present communication, a successful attempt has been made to develop a novel, simple and precise kinetic method for the trace level determination of D-PA in various samples. It is based on the inhibitory effect of D-PA on the rate of Hg(II) catalyzed substitution of CN^- in $[Ru(CN)_6]^{4-}$ by NRS. This method may be helpful for the routine quality control analysis of pharmaceutical products and for the analysis of biological samples containing D-PA.

2. Experimental

2.1. Reagents

All the reagents used were of analytical grade and double distilled water was used throughout the study. 1.0×10^{-2} M stock solutions of $K_4[Ru(CN)_6]\cdot 3H_2O$, nitroso-R-salt, $HgCl_2$ and D-PA were prepared by dissolving their appropriate amounts in water. To prevent photodecomposition, $K_4[Ru(CN)_6]\cdot 3H_2O$ solution was kept in dark amber color volumetric flask. The working solutions of each reagent, as required, were prepared by appropriate dilution from the stock solutions. Potassium dihydrogen phosphate–NaOH buffer of pH 7.00 ± 0.02 was prepared according to the literature method [68].

2.2. Instrumentation

A UV–visible spectrophotometer, model DIG SPEC-110D (Sisco Server Science Ltd., India), fitted with a self designed double walled water circulating thermostated cell compartment, was used for the spectral and absorbance measurements. For pH measurements, a Toshniwal digital pH meter model CL46 was used. The standard BDH buffers of pH 4, 7 and 10 were used to standardize the pH meter at regular intervals. The quartz cuvettes were cleaned by immersing them in 10% HNO_3 and finally with distilled water. They were also cleaned with acetone after a few kinetic runs for removing the deposited purple red colored $[Ru(CN)_5NRS]^{3-}$ complex. Remi ultra cryostat was used to maintain the temperature of the reaction system through water circulation.

2.3. General procedure

The concentration of Hg(II) was taken as 3.0×10^{-5} M to get significant change in the absorbance values. All the working solutions of the reactants and buffer were first placed in the thermostat maintained at 45.0 ± 0.1 °C, for 30 min prior to their use, to attain thermal equilibrium. Each of the solutions i.e. 2.0 mL of each; NRS, buffer, $HgCl_2$ and D-PA were pipetted out and mixed in a 10 mL volumetric flask which was also placed in the same thermostat at 45.0 ± 0.1 °C. The reaction was initiated by adding 2 mL of $K_4[Ru(CN)_6]$ solution to the reaction mixture. Then the reaction mixture was quickly shaken and transferred immediately into a 10 mm spectrophotometric cuvette placed in the thermostated cell compartment of the spectrophotometer at 45.0 ± 0.1 °C. As shown in Fig. 1, there was no interference between the absorbances of the product and the reactants. Hence, the progress of the reaction was followed by measuring the increase in the absorbance due to the formation of the substituted complex $[Ru(CN)_5NRS]^{3-}$ at its λ_{max} 525 nm without making any correction for the absorbance of reactants. The fixed time procedure as a measure of initial rate, was used to record the decrease in the absorbance of the product as a function of increased concentration of D-PA.

3. Results and discussion

3.1. The indicator reaction

The detailed kinetics and mechanism of Hg(II) catalyzed substitution of coordinated cyanide in hexacyanoruthenate(II) by NRS has recently been reported by us [61] Hence, only a brief description is presented here. However, the details related to the inhibitory action of D-PA is presented in the form of expressions to justify the direct relationship between the initial rate of reaction and the [D-PA]. The reaction proceeds at 45 °C leading to the formation of purple–red colored complex, $[Ru(CN)_5NRS]^{3-}$, having absorption maximum i.e. λ_{max} at 525 nm corresponding to the metal to ligand charge transfer (MLCT) transition. It has been found that D-PA containing sulfur is a soft base while Hg(II)

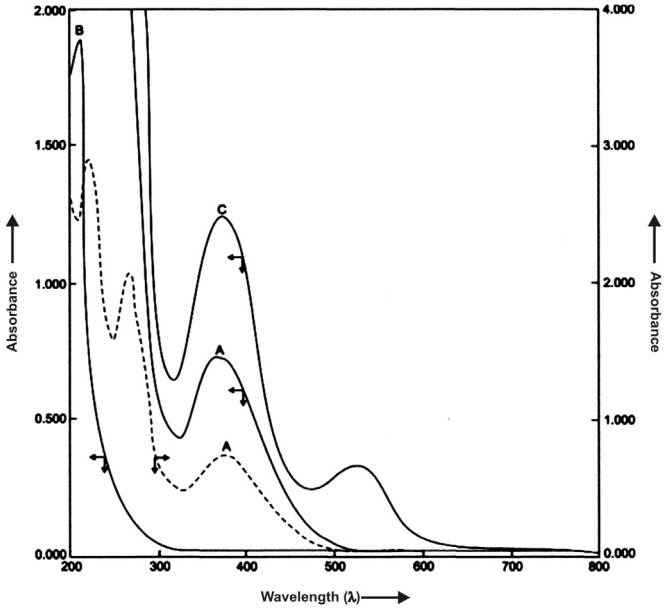


Fig. 1. UV–visible absorption spectra of the reactants and the product under the conditions: (A) [nitroso-R-salt] = 1.0×10^{-4} M, (B) $[Ru(CN)_6^{4-}] = 1.25 \times 10^{-5}$ M, (C) Product [$Ru(CN)_5$nitroso-R-salt^{3-}] formed by mixing $[Ru(CN)_6^{4-}] = 3.3 \times 10^{-3}$ M, [nitroso-R-salt] = 1.0×10^{-3} M, [Hg(II)] = 2.0×10^{-4} M, μ = 0.1 M (KCl), pH = 7.00 ± 0.02 and temperature = 45.0 ± 0.1 °C.

is a soft acid. Therefore, D-PA easily complexes with the catalyst Hg(II) and thus inhibits the catalyzed reaction between $[Ru(CN)_6]^{4-}$ and NRS. The inhibition of the catalyzed reaction rate by sulfur containing drug i.e. D-PA in the present case has been attributed to the formation of stable complex of stoichiometry 1:1 between Hg(II) catalyst and D-PA inhibitor [69]. The catalyst-inhibitor complex resulted in the decrease of free catalyst concentration i.e. [Hg(II)] which ultimately masks the catalytic activity of Hg(II) catalyst. The inhibitory effect depends upon the [D-PA]/[Hg(II)] ratio in the indicator reaction system [62,63]. As the concentration of the inhibitor was increased in the indicator reaction system, the reaction rate started decreasing simultaneously.

The UV–visible spectra of the reactants and the product are shown in Fig. 1. It is clear from Fig. 1 that there is no interference between the absorbances of product and those of reactants. Hence, the progress of reaction was followed by measuring the decrease in absorbance at 525 nm (λ_{max} of the product $[Ru(CN)_5NRS]^{3-}$) with the increased concentration of the inhibitor D-PA. The decrease in the rate of indicator reaction is directly proportional to the concentration of the inhibitor i.e. [D-PA] added to the reaction system. This observation formed the basis for the linear relationship between the initial rate of the indicator reaction and the concentration of the inhibitor D-PA and was utilized for D-PA determination in the real samples (tablets).

3.2. Calibration graph for the determination of D-penicillamine

For the determination of D-PA, the fixed time absorbance (A_t) was recorded as a measure of initial rate. The plots of absorbance (A_t) versus [inhibitor] i.e., [D-PA] were found to be linear as shown in Fig. 2 which served as calibration curves. The linear regression equations obtained provided the basis for the determination of D-PA. The expressions relating the initial rate i.e. the absorbance change at a fixed time A_t (t = 15 and 20 min) to the concentration of inhibitor, D-PA, in the reaction mixture are shown in Eqs. (1) and (2).

$$A_{15} = 0.119 - 3.95 \times 10^4 [\text{D-PA}] \quad (1)$$

$$A_{20} = 0.149 - 4.84 \times 10^4 [\text{D-PA}] \quad (2)$$

The coefficients of the determination corresponding to the calibration Eqs. (1) and (2) are 0.9996 and 0.9981, respectively,

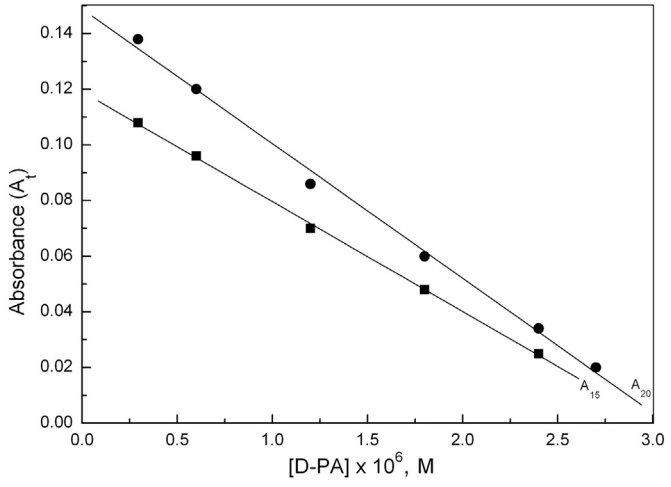
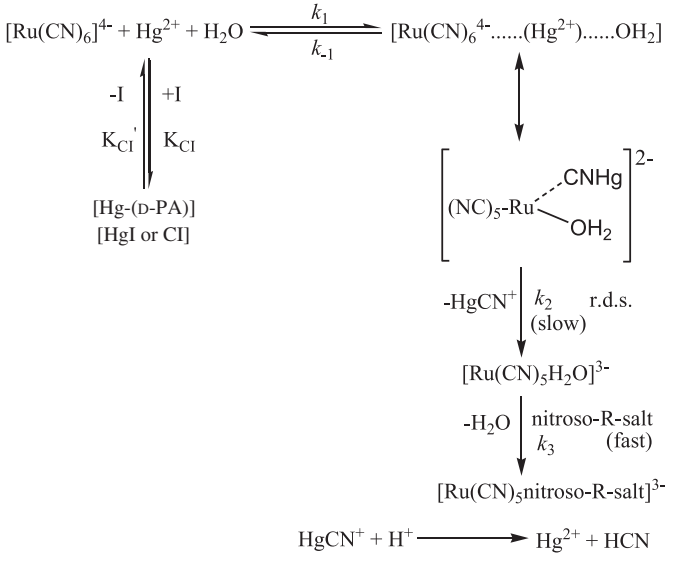


Fig. 2. Calibration curves for the determination of D-penicillamine at $[Ru(CN)_6^{4-}] = 8.75 \times 10^{-5}$ M, [nitroso-R-salt] = 3.5×10^{-4} M, [Hg(II)] = 3.0×10^{-5} M, μ = 0.1 M (KCl), pH = 7.00 ± 0.02 and temperature = 45.0 ± 0.1 °C.

which showed an excellent correlation between the initial rate (fixed time procedure) and [D-PA]. To judge the precision and accuracy of the method, the recovery experiments were performed where D-PA was determined in aqueous solutions in the range $0.294–2.70 \times 10^{-5}$ M. The determined amounts, along with the corresponding standard deviations and percentage errors are given in Table 1. The detection limit was found to be 2.5×10^{-7} M.

3.3. Mechanism of inhibition

The mechanism of inhibition caused by D-PA i.e. inhibitor (I) to the Hg(II) catalyzed cyanide substitution of $[Ru(CN)_6]^{4-}$ by NRS with a simple modification (which now involves inhibitor) to our earlier proposed mechanistic scheme for the reaction system without inhibitor [60] is given as follows:



The uncatalyzed substitution reaction of $[Ru(CN)_6]^{4-}$ is extremely slow and is almost negligible in the present case [60]. Hence, uncatalyzed reaction in the proposed mechanistic scheme involving inhibitor D-PA has not been taken into consideration while deriving the rate equation. The substrate $[Ru(CN)_6]^{4-}$ present in excess is denoted by 'S' while its initial concentration by $[S_0]$. Considering this indicator reaction as an enzyme catalyzed reaction involving single substrate, the rate of reaction was deduced using Lineweaver–Burk Eq. i.e. double reciprocal [70] where V_0 indicates the initial rate of the catalyzed reaction and V_{max} is the maximum attainable rate at a particular concentration of the catalyst Hg(II) and large excess concentration of the substrate, $[Ru(CN)_6]^{4-}$. K_m is Michaelis–Menten constant involving a single

Table 1
Quantitative determination of D-PA under the reaction conditions: $[Ru(CN)_6^{4-}] = 8.75 \times 10^{-5}$ M, [NRS] = 3.50×10^{-4} M, $[Hg^{2+}] = 3.0 \times 10^{-5}$ M, μ = 0.1 M (KCl), pH = 7.00 ± 0.02 and temperature = 45.0 ± 0.1 °C.

[D-PA] × 10^6 M (taken)	A_{15}		A_{20}	
	[D-PA] × 10^5 M (found)	% error	[D-PA] × 10^5 M (found)	% error
0.29	0.29 ± 0.003	0.00	0.31 ± 0.021	+6.90
0.60	0.60 ± 0.015	0.00	0.60 ± 0.010	0.00
1.20	1.25 ± 0.050	+4.2	1.30 ± 0.030	+8.33
1.80	1.80 ± 0.036	0.00	1.86 ± 0.032	−3.33
2.40	2.40 ± 0.029	0.00	2.40 ± 0.041	0.00
2.70	–	–	2.70 ± 0.025	0.00

substrate [70,71]. Thus, the rate equation in the presence of inhibitor is given by Eq. (3).

$$\frac{1}{V_0} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \frac{1}{[S_0]} \tag{3}$$

The rate of the indicator reaction in the presence of inhibitor D-PA can be expressed as in Eq. (4) where K'_m is apparent Michaelis–Menten constant [72] as given in Eq. (5).

$$V_i = \frac{V_{max}}{1 + \frac{K'_m}{[S_0]}} \tag{4}$$

$$K'_m = K_m \left(1 + \frac{[I_0]}{K'_{CI}}\right) \tag{5}$$

After substituting K'_m from Eq. (5) into Eq. (4) results in Eq. (6) where I_0 is the initial concentration of the inhibitor i.e. D-PA, V_i denotes the rate of the indicator reaction in the presence of inhibitor D-PA while the catalyst concentration was kept constant and K'_{CI} is the dissociation constant of catalyst-inhibitor (CI i.e. Hg-(D-PA) complex.

$$V_i = \frac{V_{max}}{1 + \frac{K_m}{[S_0]}\left(1 + \frac{[I_0]}{K'_{CI}}\right)} \tag{6}$$

The indicator reaction rate Eq. (6), which in presence of the inhibitor, has been transformed to Linewaver–Burk's form as shown in Eq. (7).

$$\frac{1}{V_i} - \frac{1}{V_{max}} = \frac{K_m}{V_{max}[S_0]} + \frac{K_m}{V_{max}[S_0]} \frac{[I_0]}{K'_{CI}} \tag{7}$$

The V_{max} used in Eq. (7) is not an experimental quantity. However, it is evaluated from the intercept of the plot of $\frac{1}{V_0}$ versus $\frac{1}{[S_0]}$ using Eq. (3) in the absence of the inhibitor as shown in Fig. 3. Then the slope of this plot gave the value of K_m as 0.59 mM. For the validity and good results from Eq. (7), inhibitor 'I' must form a complex of the type (CI) with catalyst 'C'. There should be no substrate inhibition i.e. substrate-inhibitor complex should not be formed.

The plot of $(\frac{1}{V_i} - \frac{1}{V_{max}})$ versus $[I_0]$ i.e. at different concentration of the inhibitor D-PA was found to be linear as shown in Fig. 4. From the intercepts of this plot, the value of K_m in the presence of inhibitor D-PA was evaluated and was found to be in good agreement with its calculated value using $\frac{1}{V_0}$ versus $\frac{1}{[S_0]}$ plot (Fig. 3). The value of K'_{CI} corresponding to the Hg-(D-PA) complex was calculated from the slope of the plot as 3.46×10^{-6}.

3.4. Interference studies

The interferences caused due to the presence of different cations, anions and organic molecules were tested and found that most of the cations like Sn^{2+}, Pb^{2+}, Sr^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, Fe^{3+}, Cr^{3+}, Al^{3+} and anions like Br^-, I^-, NO_3^-, $C_2O_2^{2-}$ do not interfere in the determination of D-PA in the present indicator reaction system except Co^{2+}, Na^+, SO_4^{2-}, $S_2O_3^{2-}$ and TGA [61]. The interference caused by Co^{2+} ions may be due to their salt formation with NRS. The interference by TGA may be due to their complex forming tendencies with Hg(II) based the HSAB principle [64] and hence reducing its catalytic activity while interference due to Na^+ ions needs further studies for explanation. Sodium salts of some aminopolycarboxylates like EDTA, IDA, NTA, and HEDTA also interfere to some extent which may also be due to the presence of free Na^+ ions in their solutions.

It is important to mention here that no complexing agent i.e. ligand which forms a complex of high stability with Hg(II) should be present in the indicator reaction system for the accuracy in the determination of D-PA. In addition, the metals that complex with D-PA more stronger than Hg(II) under the present experimental conditions must also be absent in the indicator reaction system.

3.5. Application of the method

In order to evaluate the applicability and validity of the proposed method for the analysis of real samples, the method was applied for the analysis of commercially available pharmaceutical samples of D-PA and the results were compared with United States Pharmacopeia (USP) method [73] which showed good precision (Table 2).

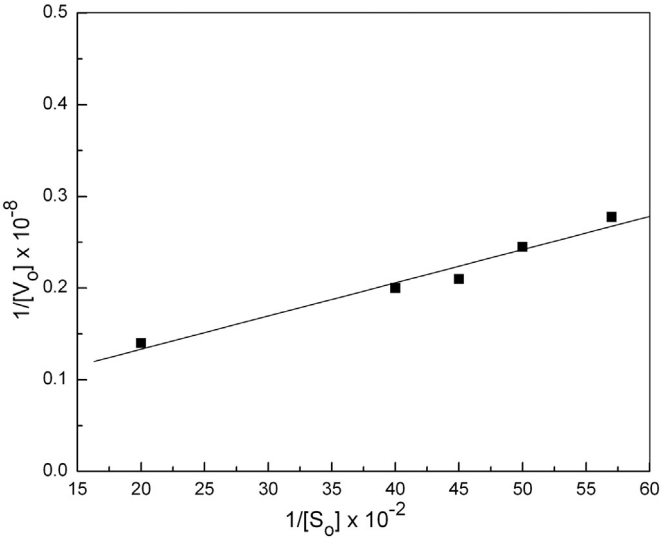


Fig. 3. The plot of $\frac{1}{V_0}$ versus $\frac{1}{[S_0]}$ for the determination of V_{max} by variation of substrate concentration in absence of inhibitor at [Hg(II)] = 3.0×10^{-5} M, [nitroso-R-salt] = 3.5×10^{-4} M, pH = 7.00 ± 0.02, temperature = 45.0 ± 0.1 °C and μ = 0.1 M (KCl).

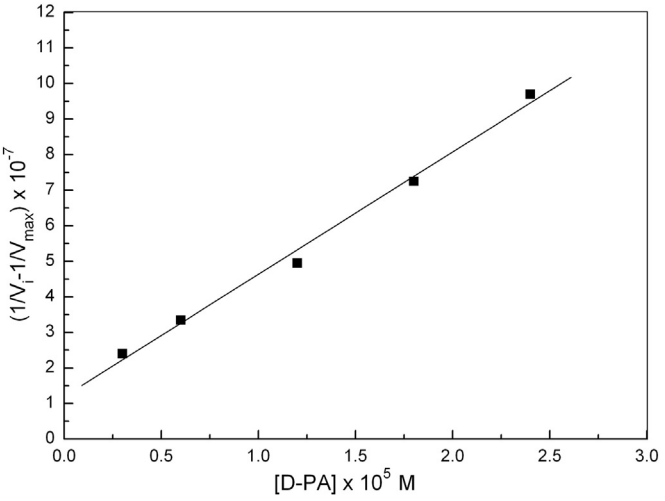


Fig. 4. The plot of $(\frac{1}{V_i} - \frac{1}{V_{max}})$ versus $[I_0]$ for determination of K'_{CI} and K_m in the presence of inhibitor D-penicillamine at [Hg(II)] = 3.0×10^{-5} M, [nitroso-R-salt] = 3.5×10^{-4} M, pH = 7.00 ± 0.02, temperature = 45.0 ± 0.1 °C and μ = 0.1 M (KCl).

Table 2
Application of the proposed method for the determination of D-PA in D-PA tablets and the recovery data under optimized reaction conditions: $[Ru(CN)_6^{4-}] = 8.75 \times 10^{-5}$ M, [nitroso-R-salt] $= 3.5 \times 10^{-4}$ M, [Hg(II)] $= 3.0 \times 10^{-5}$ M, $\mu = 0.1$ M (KCl), pH = 7.00 ± 0.02 and temperature = 45.0 ± 0.1 °C.

Sample D-PA tablet	Labeled value (mg/tablet)	Amount found (mg/tablet)	Mean recovery ± SD (%)	
			Proposed method	USP method [73]
Tablet 1	250	253.50	101.4 ± 1.4	100.5
Tablet 2	250	249.10	99.6 ± 1.4	99.4
Tablet 3	250	250.61	100.2 ± 2.0	100.1
Tablet 4	250	251.17	100.5 ± 1.1	100.7
Tablet 5	250	249.48	99.8 ± 1.8	99.6

4. Conclusion

The kinetic method reported here for the determination of D-PA is more accurate and sensitive because the uncatalyzed reaction is insignificant in the present system and the presence of inhibitor directly affects the rate of the catalyzed reaction. The detection limit was found to be 2.5×10^{-7} M which shows that the proposed analytical method can be successfully adopted for D-PA determination at such a low level. To the best of our knowledge, this is the first kinetic method for the determination of D-PA at such a low level using the indicator reaction between $[Ru(CN)_6]^{4-}$ and NRS in presence of Hg(II) as a catalyst. This method is simple, easy and inexpensive in comparison to the other sophisticated methods [8,10,11,17,31–38,42–53] available in literature and can be adopted for the routine quality control analysis of pharmaceutical products and for the analysis of biological samples containing D-PA.

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