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Short communication

Fluorometric assay of tiopronin based on inhibition of multienzyme redox system

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Abstract

In this paper, a simple and sensitive fluorimetric method for the determination of tiopronin (*N*-(2-mercaptopropionyl)-glycine) is proposed. The method is based on the strong inhibitory effect of tiopronin on the multienzyme redox system of hemoglobin, nicotinamide adenine dinucleotide (NADH) and H_2O_2, in which the intrinsic fluorescence of NADH was employed as the detection signal. The calibration graph is linear in the range 6.13×10^{-7} to 6.13×10^{-6} M with a detection limit of 1.65×10^{-7} M and the relative standard deviation of 2.02%. Kinetics in the pseudo-first-order conditions was investigated by stopped-flow spectrofluorometry and the inhibition mechanism of tiopronin was verified of the competitive type.

Keywords: Fluorometric assay; Tiopronin; Inhibition; Multienzyme redox system

1. Introduction

Nicotinamide adenine dinucleotide (NADH) is an important biological molecule that participates in many metabolic processes. It is generally known that NADH plays a central role in the multienzyme redox system in mitochondrial electron transport chain [1,2]. In this work, we first observed a strong inhibitory effect of tiopronin on the multienzyme redox system as follows:

$$H_2O_2 + NADH + H^+ \xrightarrow{Hb[O]} NAD^+ + 2H_2O$$

in which hemoglobin (Hb) was used as mimetic enzyme instead of horseradish peroxidase (HRP) [3].

Tiopronin is a synthetic antioxidant used in clinical applications. It is effective in the treatment of cystinuria, rheumatoid arthritis, as well as hepatic disorders [4–6]. Recent studies have shown that it may act as a free radical scavenger because of its thiol group [7]. It can also increase intracellular concentration of GSH and other non-protein sulphydryl groups and thereby increase intracellular defense against oxygen free radicals [8]. A number of studies on the determination of tiopronin using spectrophotometry [9,10], chemiluminescence [6,11], fluorimetry [12,13], amperometry [14] and liquid chromatography [15–17] have been published.

Fluorimetric analysis is widely used for its high sensitivity, but no work has been reported on the fluorometric determination of tiopronin based on an enzymatic reaction. In this paper, we developed a simple and sensitive fluorimetric method for determining tiopronin based on its inhibitory effect on the multienzyme redox system mentioned above. The calibration graph is linear in the range 6.13×10^{-7} to 6.13×10^{-6} M with a detection limit of 1.65×10^{-7} M and R.S.D. of 2.02%. This method is superior to the others in literature in some ways. First, the sensitivity of this method is higher than that of other indirect fluorimetric methods [12,13]. Second, the intrinsic fluorescence of NADH was employed as the detection signal and no tedious derivation was needed, which greatly simplified the operation. Being free of organic dyes and poisonous substance, this method is environmental-friendly. Third, the reaction medium is mild, in physiological condition, which is more practical for bioactive substance such as tiopronin.

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The inhibition mechanism of tiopronin on the multienzyme redox system was investigated by stopped-flow spectrofluorometry.

2. Experimental

2.1. Reagents

Hemoglobin (bovine erythrocytes) solution was prepared by dissolving certain amount of Hb (Shanghai Institute of Biochem., Shanghai, China) in 0.05 M Tris–HCl–EDTA buffer (0.05 M Tris–HCl containing 0.1 mM EDTA), pH 8.50 and stored below 4 °C. Hydrogen peroxide solution was prepared by diluting 0.01 ml of 30% H_2O_2 (standardized by titration with $KMnO_4$ standard solution) to 100 ml. It was stored in a brown bottle in a refrigerator. Stock solution of 1.00×10^{-2} M tiopronin (TP; Sigma) was prepared by dissolving in double distilled water and stored at 4 °C. This solution was diluted appropriately before use. NADH, disodium salt (Sigma) solution was made in the concentration of 1.00×10^{-3} M with Tris–HCl–EDTA buffer (pH 8.5) daily. The tiopronin-containing drugs, Kaixilai tablets and injection formulation were purchased from Xinyi Pharmaceutical Company, Henan, China. Double distilled water was used throughout. All other chemicals were of analytical-reagent grade.

2.2. Apparatus

A LS55 Fluorescence spectrophotometer (Perkin-Elmer) was used with a 1 cm quartz cell to record the relative fluorescent intensity. Kinetic experiments were performed on an SX-18MV stopped-flow analyzer (Applied Photophysics Ltd., UK). The experimental temperature, for both quantitative determination and kinetic researches, was controlled with a TB-85 thermostat water bath (Shimadzu Kyoto, Japan). For pH measurement, a TOA Electronics Model PHS-3C precision pH meter (Shanghai, China) was used.

2.3. Procedures

2.3.1. Fluorescent measurements

Each colorimetric tube was orderly filled with 400 μl 1.00×10^{-4} M H_2O_2, 100 μl 1.00×10^{-3} M NADH, a proper amount of TP solution and 400 μl 5.00×10^{-6} M Hb, and then diluted with pH 8.5 Tris–HCl–EDTA buffer solution to 10 ml. Contained in a thermostatic water bath (30 ± 0.2 °C) for 10 min, NADH fluorescence intensity was monitored at excitation wavelength of 340.0 nm and emission wavelength of 454.0 nm. The percentage inhibition (%*I*) was calculated base on the following equation:

$$\%I = 100\frac{(F_s - F_e) - (F_s - F_i)}{F_s - F_e} = 100\frac{F_i - F_e}{F_s - F_e}$$

where F_s representing substrate fluorescence intensity alone; F_i, substrate fluorescence in the presence of Hb and inhibitor; F_e, substrate fluorescence in the presence of Hb only.

2.3.2. Stopped-flow spectrofluorometry analysis

Fast kinetic experiments were performed in an Applied Photophysics SX-18MV stopped-flow spectrofluorometer equipped with a high-intensity xenon arc lamp. The excitation/emission wavelength was 342/468 nm and slit width 3 nm. The temperature was controlled at 30 ± 0.2 °C. Time courses of reactions were recorded. All data were determined from averages of three individual traces and concentrations after mixing were stated.

3. Results and discussion

3.1. Spectra characteristics of the reaction

The intrinsic fluorescence of NADH was monitored in this work to indicate extent of reaction. We observed that relative fluorescence intensity (RFI) of NADH decreased upon adding Hb and H_2O_2, but remarkably restored in the presence of ultra trace of TP. Thus, it is conceivable that TP acts as an inhibitor to this multienzyme redox system. Furthermore, there is good linearity between concentration of TP and %*I* in certain range, on which a new method for the determination of TP is based.

The 3D fluorescent spectra of the reactions were obtained and shown in Fig. 1. It is noted that both in the absence and presence of TP, the spectra were similar in profile but different in size. Addition of TP simply resulted in the inhibitory effect on Hb activity and no new fluorescent product was observed.

3.2. Inhibition mechanism

In order to elucidate the inhibition mechanism, stopped-flow kinetic studies were carried out. Time courses of reactions were recorded with various concentrations of NADH and TP, and initial rates of the reactions were obtained from each reaction curve. According to the method of Lineweaver–Burk, double reciprocal plots of $1/v$ versus $1/[S]$ were done, and the details are shown in Fig. 2. Increasing slopes were observed but intercepts remained the same in Lineweaver–Burk plots with adding of inhibitor, which confirmed the inhibition of TP on Hb was of the competitive type.

3.3. Optimization of variables

The pH-dependence system was investigated over the range of pH 7.4–9.1 using Tris–HCl–EDTA buffer. The data are shown in Fig. 3. It can be seen the %*I* increases with pH value up to 8.80, and there exists a platform on the curve within range of pH 8.50–8.80. Hence, the Tris–HCl–EDTA buffer with pH 8.50 was chosen in the subsequent experiments. As thiols are easily oxidized with dissolved oxygen in alkaline media and oxidation is catalytically accelerated

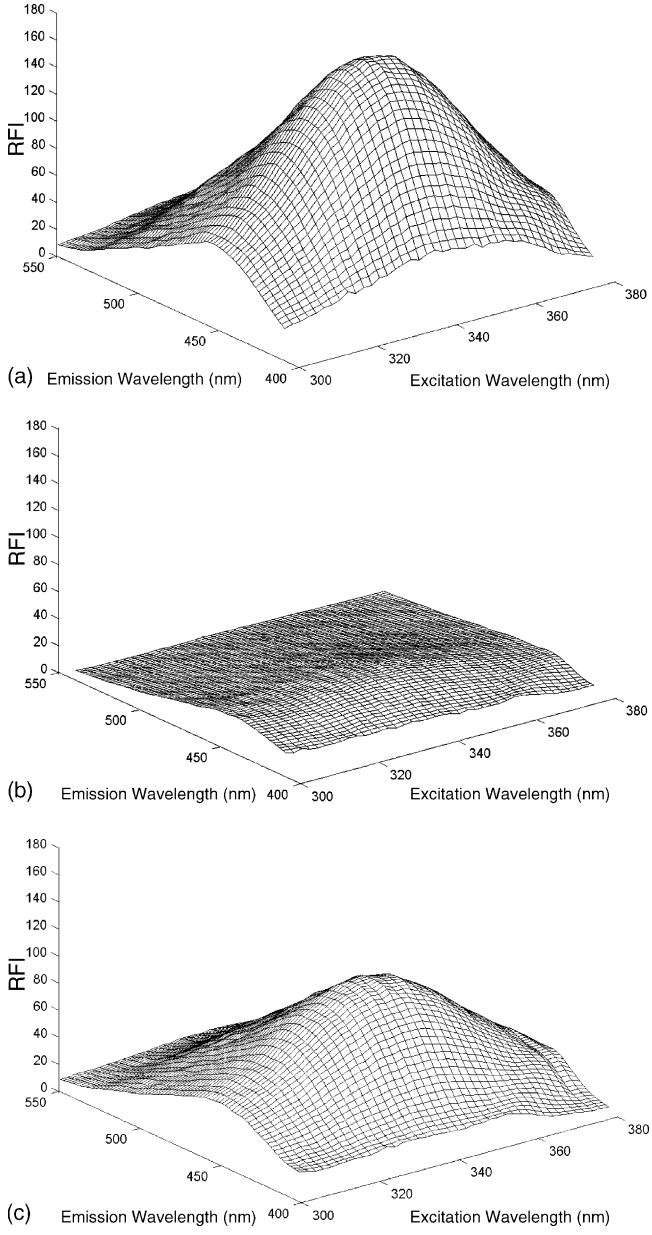

Fig. 1. 3D fluorescence spectra of the reaction: (a) in the absence of Hb and TP; (b) in the presence of Hb; (c) in the presence of Hb and TP. *Conditions*: 4.00×10^{-6} M H_2O_2, 1.00×10^{-5} M NADH, 2.00×10^{-7} M Hb and 5.00×10^{-6} M TP in 0.05 M pH 8.5 Tris–HCl–EDTA buffer, 30 °C.

with various metals [18], 0.1 mM EDTA–2Na was added to buffers to eliminate the interference.

The effect of temperature on the system was investigated in a range from room temperature up to 50 °C. The time needed to reach equilibrium, no more than 10 min, was prolonged with the decreasing temperature (data not shown). Given decomposition of H_2O_2, temperature was kept at 30 °C and FI measured after 10 min.

The optimal reagent concentrations were studied and the recommended values are summarized in Table 1. It is noted that the percent inhibition of TP decreases from 70 to 40% with the Hb concentration increasing from 5.00×10^{-8} to

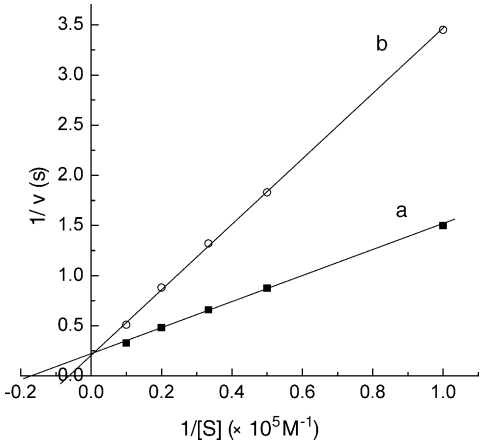

Fig. 2. Lineweaver–Burk plots in the presence of (a) 0 and (b) 5.00×10^{-6} M of TP. NADH concentrations: 1.00×10^{-5}, 2.00×10^{-5}, 3.00×10^{-5}, 5.00×10^{-5} and 10.00×10^{-5} M in 0.05 M pH 8.5 Tris–HCl–EDTA buffer, 30 °C.

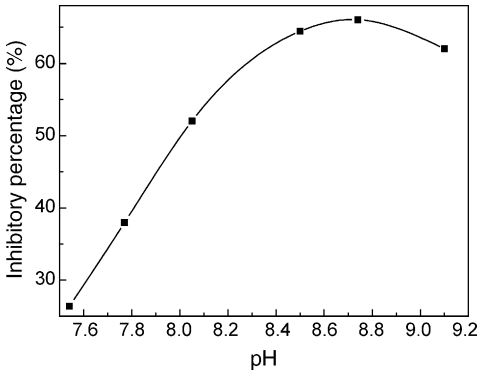

Fig. 3. Effect of pH on the percent inhibition of TP on Hb activity. *Conditions*: 4.00×10^{-6} M H_2O_2, 1.00×10^{-5} M NADH, 2.00×10^{-7} M Hb and 5.00×10^{-6} M TP in 0.05 M Tris–HCl–EDTA buffer, 30 °C.

5.00×10^{-7} M, i.e. the inhibitor has less effect involving higher concentrations of Hb. This is often seen in enzyme-inhibition reactions. Besides, the reproducibility of the signal becomes poor with higher Hb concentration. Considering the sensitivity as well as the reproducibility, 2.00×10^{-7} M of Hb was recommended in the following determination.

As for the substrate, the percent inhibition of TP decreased greatly with increasing NADH concentration, especially in the range from 5.00×10^{-6} to 4.00×10^{-5} M (see Fig. 4). Considering the fluorescence intensity getting too weak at very low NADH concentration, 1.00×10^{-5} M NADH was chosen for further study. The %*I* increased with the increase

Table 1
Optimum condition for tiopronin determination

Variable	Range studied	Recommended value
pH	7.4–9.1	8.5
Hb (M)	$0.50–5.00 \times 10^{-7}$	2.00×10^{-7}
NADH (M)	$0.50–8.00 \times 10^{-5}$	1.00×10^{-5}
H_2O_2 (M)	$0.10–10.0 \times 10^{-6}$	4.00×10^{-6}
Temperature (°C)	20–50	30

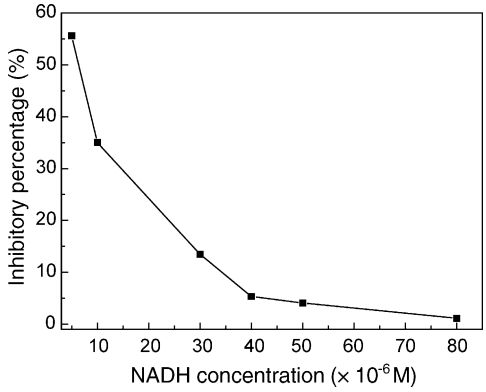

Fig. 4. Effect of NADH concentration on the percent inhibition of TP on Hb activity. *Conditions*: 4.00×10^{-6} M H_2O_2, 2.00×10^{-7} M Hb and 5.00×10^{-6} M TP in 0.05 M pH 8.5 Tris–HCl–EDTA buffer, 30 °C.

in H_2O_2 up to 4.00×10^{-6} M, above which it had little effect. So 4.00×10^{-6} M H_2O_2 was used in the recommended procedure.

3.4. Analytical characteristics

The calibration graph is linear over the TP concentration (c) range 6.13×10^{-7} to 6.13×10^{-6} M, which obeyed the equation as follows:

$$\%I = (-0.9050 \pm 0.2314) + (3.6250 \pm 0.0496) \times (10^7 \times c)$$

$$r = 0.996,\ n = 9$$

where "c" is the concentration of TP. The detection limit, calculated according to the $3 \times Sb/k$ criterion, was found to be 1.65×10^{-7} M. The relative standard deviation for 11 replicate determination of 2.50×10^{-6} M TP was 2.02%.

3.5. Interference

To indicate selectivity of the proposed method, IC_{50} values (defined as the concentration of inhibitor required to inhibit 50% of the Hb activity under the assay conditions) of several amino acids, reducing compounds, vitamins and metal ions were determined, and results were compared with the IC_{50} of TP. In the mean time, in order to assess a possible involvement of reactive oxygen species (ROS) in this procedure, the influences of several scavengers for $O_2^{\bullet -}$ and $^{\bullet}OH$ on the determination were also investigated. The results in Table 2 show a fairly satisfactory selectivity of the method. Especially, the IC_{50} values of methanol and DMSO are about 10^5 times as high as that of TP, while IC_{50} of SOD is 18.0 U/ml, which suggested that $O_2^{\bullet -}$ may probably be involved in the reaction.

3.6. Applications

The method was applied to determine tablets and injections containing TP. For analysis of tablets, accurate amount of powdered tablets were dissolved in double distilled water and then the solution was filtered into a 100-ml calibrated flask to obtain a concentration of ca. 1 mg ml^{-1}. These solutions were diluted appropriately with Tris–HCl–EDTA buffer before use. In order to evaluate the validity of the proposed method, HPLC method was also used for the determination according to the procedure described in literature [15]. The results obtained by the two methods were compared by Student's t-test. No significant differences were found between them (details shown in Table 3). Recovery tests were also run on each of the analyzed samples by adding a known amount

Table 2
IC_{50} of TP and foreign species in the proposed method

Substance	IC_{50} (M)	Substance	IC_{50} (M)	Substance	IC_{50} (M)
TP	4.52×10^{-6}	Thiamine	2.79×10^{-2} [a]	Threonine	1.12×10^{-2}
Methanol	5.50×10^{-1}	Fe^{3+}	3.10×10^{-4} [a]	L-Cysteine	2.67×10^{-4}
DMSO	7.80×10^{-1}	Ca^{2+}	3.38×10^{-2}	Glycin	2.42×10^{-1}
SOD	18.0[b]	Mg^{2+}	1.71×10^{-2}	Tyrosine	1.83×10^{-4} [a]
Glucose	2.94×10^{-1}	Zn^{2+}	1.35×10^{-2}	L-Tryptophan	2.87×10^{-3} [a]
Glutathion	1.40×10^{-4}	Ascorbate	2.20×10^{-2}	L-Phenylalanine	2.68×10^{-2}
BSA	8.83×10^{-4}	ATP–Na_2	2.08×10^{-2} [a]	L-Glutamine	1.12×10^{-2} [a]

[a] The substance enhances the reaction by 50% in this concentration.
[b] SOD activity (U/ml).

Table 3
Determination and recovery test of tiopronin in pharmaceutical preparations

Samples	Amount (mg)					Recovery test[a]	
	Label	Found ± S.D. (n = 9)		t[c] (P > 0.05)	Added (mg)	Recovered (mg)	Recovery ± S.D. (%)
Tablets	100	98.2 ± 2.1[a]	97.6 ± 2.5[b]	1.26	25.0	23.9	95.6 ± 2.3
Injection	100	101.0 ± 1.3[a]	99.8 ± 0.9[b]	1.35	25.0	25.5	102.1 ± 1.9

[a] Proposed method.
[b] HPLC method according to literature [15].
[c] Theoretical value = 2.101, n = 9 with 95% confidence limits.

of TP standard to the samples before the recommended procedure. As shown in Table 3, the recoveries were 95.6 and 101.2%, indicating that the method is reliable for the determination of TP in pharmaceutical preparations.

4. Conclusions

The inhibitory effect of tiopronin on the multienzyme redox system was applied to the determination of tiopronin in this paper. The proposed method has high sensitivity and precision. Being free of tedious derivation and poisonous substance, the method is simple and environmental-friendly. The utility of the method was illustrated by the analysis of commercial pharmaceutical preparations.

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