Journal of Fluorescence, Vol. 13, No. 2, March 2003 (© 2003)

Fluorometric Determination of 3,4-Dihydroxyphenylalanine with 2-Cyanoacetamide

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Received September 6, 2002; revised October 11, 2002; accepted October 11, 2002

A new method has been developed for the fluorometric determination of 3,4-dihydroxyphenylalanine on the basis of the reaction with 2-cyanoacetamide. The reaction product has excitation and emission peaks at 335 and 405 nm, respectively. Under the optimum conditions, the fluorescence intensity is a linear function of 3,4-dihydroxyphenylalanine concentration between 2.5×10^{-8} and 2.0×10^{-6} mol/L. The detection limit, corresponding to a signal-to-noise ratio of 3, is 6.8×10^{-9} mol/L. Most amino acids, epinephrine, and norepinephrine do not interfere with the determination of 3,4-dihydroxyphenylalanine. The proposed method has been successfully applied to the determination of 3,4-dihydroxyphenylalanine in pharmaceutical preparations.

KEY WORDS: 3,4-Dihydroxyphenylalanine; 2-cyanoacetamide; fluorimetry.

INTRODUCTION

L-3,4-Dihydroxyphenylalanine is also called L-dopa. It is a forerunner of L-dopamine and is employed in the treatment of Parkinson's disease, which is caused by a lack of dopamine in brain tissues. Because 3,4-dihydroxyphenylalanine can permeate into brain tissue and then be converted into dopamine in the presence of decarboxylase, it is important to determine the amount of 3,4-dihydroxyphenylalanine and its metabolites in biological and pharmaceutical preparations. High-performance liquid chromatography (HPLC) [1,2], spectrophotometry [3–6], and chemiluminence methods [7] are used for the determination of the aromatic amino acids. Fluorimetric determinations of 3,4-dihydroxyphenylalanine have been reported [8–11]. These determinations used redox reactions [8] and derivatization methods [9], but the sensitivities of these methods are low, and interferences are serious problems. To raise the sensitivity, a fluorescence probe (Tb^{3+}) method for the determination of 3,4-dihydroxyphenylalanine was proposed [10], which is based on the fact that at pH = 8.0–8.5, 3,4-dihydroxyphenylalanine can complex with Tb^{3+}, with emission of the characteristic fluorescence of Tb^{3+}. The sensitivity of this method is high, the detection limit is 7.5×10^{-9} mol/L (signal-to-noise ratio [S/N] = 3), but the interferences from epinephrine (E) and norepinephrine (NE) are serious problems. Our experiments indicated that Tb^{3+} could also complex with E and NE with the emission of Tb^{3+}, and the method has already been used for the determination of the E [12].

The experiments showed that at pH = 13.0–13.3, 3,4-dihydroxyphenylalanine could react with 2-cyanoacetamide and emit strong fluorescence. The detection limit of this method is 6.8×10^{-9} mol/L (S/N = 3), and most amino acids, E, and NE do not interfere with the determination of 3,4-dihydroxyphenylalanine. Therefore, this is a sensitive, selective method for the determination of 3,4-dihydroxyphenylanine.

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1053-0509/03/0300-0123/0

EXPERIMENTAL

Apparatus

All fluorescence measurements were made with a Shimazdu RF-540 spectrofluorimeter (Japan). All pH measurements were made with a pHs-2 acidity meter (leici, Shanghai).

Reagents and Solutions

3,4-Dihydroxyphenylalanine solution: stock solution (1.00×10^{-2} mol/L) of the 3,4-dihydroxyphenylalanine was prepared by dissolving 0.1972 g of the compound (Fluka) in water in a 100-mL calibrated flask.

2-Cyanoacetamide solution: stock solution (2%, w/v) was prepared by dissolving 1.0 g of the compound in water in a 50-mL calibrated flask.

Buffer solution: 0.1 mol/L borate–NaOH buffer solution was prepared by dissolving 3.8 g borate in 100 mL distilled water, then adjusting the pH to 13.0–13.3 with 2.0 mol/L NaOH.

L-dopa sample solution: a random sampling of four tablets L-dopa was taken. Tablets were ground to powder, 0.0986 g was weighed, dissolved in water, and diluted to 50 mL in a calibrated flask.

Procedures

To a 25-mL test tube, solutions were added in the following order. 1 mL of 0.1 mol/L borate–NaOH buffer solution (pH = 13.0–13.3), 1 mL of 3,4-dihydroxyphenylalanine solution (2.5×10^{-7} to 2.0×10^{-5} mol/L), and 1.5 mL of 2-cyanoacetamide solution (2%, w/v). The mixture was heated in a boiling waterbath for 10 min, then cooled with tap water to room temperature. The mixture was diluted to 10 mL with distilled water. The fluorescence intensity was measured in a 1-cm quartz cell at excitation and emission wavelengths of 335 and 405 nm, respectively.

RESULTS AND DISCUSSION

Fluorescence Spectra

Experiments showed that at pH = 13.0–13.3, 3,4-dihydroxyphenylalanine could react with 2-cyanoacetamide and form a fluorescent product, which emitted strong fluorescence. The excitation and emission spectra of 2-cyanoacetamide–buffer (1) and 3,4-dihydroxyphenylalanine–2-cyanoacetamide–buffer (2) are shown in Fig. 1. The fluorescent species have excitation and emission peaks at 335 and 405 nm, respectively.

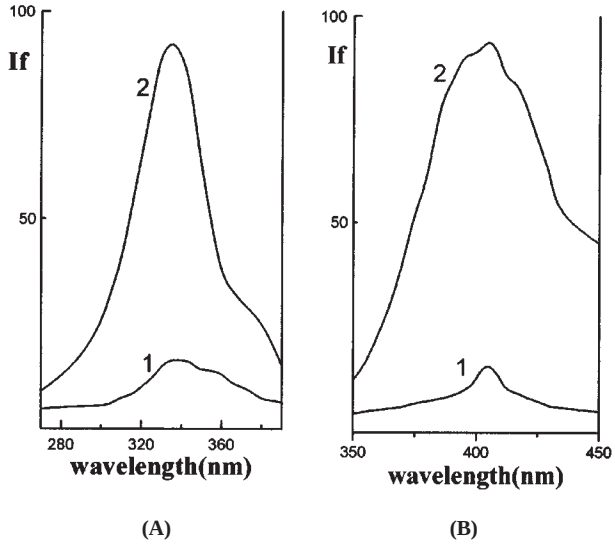


Fig. 1. Fluorescence spectra: (A) excitation spectra; (B) emission spectra. 1: 2-cyanoacetamide-borate-NaOH; 2: L-dopa–2-cyanoacetamide–borate–NaOH. Conditions: L-dopa: 5×10^{-7} mol/L; borate–NaOH buffer: 1.0 mL (pH = 13.2); 2-cyanoacetamide solution 2% (w/v): 1.5 mL.

Effect of pH and Buffer

The effect of solution pH on the fluorescence intensity of the system is shown in Fig. 2. The strongest fluorescence intensity is obtained at pH = 13.0–13.3.

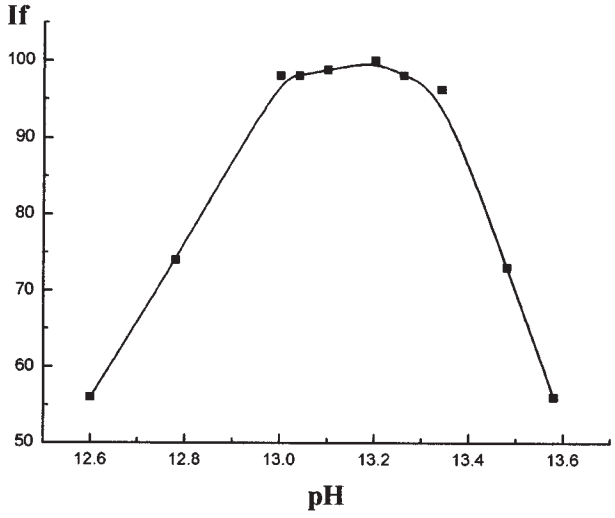


Fig. 2. Effect of pH. Conditions: L-dopa: 5×10^{-7} mol/L; borate–NaOH buffer: 1.0 mL (pH = 13.2); 2-cyanoacetamide solution 2% (w/v): 1.5 mL.

The type of buffer greatly affects the fluorescence intensity. In this article, the following buffers were tested: borate–NaOH, Na_2HPO_4, NH_3–NH_4Cl, and 0.1 mol/L NaOH. The results of the tests shown in Table I indicate that borate–NaOH is the best of the buffers tested and the optimum volume of buffer is 1.0 mL.

Effects of Temperature and Heating Time

The effects of temperature and heating time on the fluorescence intensity of the system were studied and are shown in Fig. 3. At low temperature, the fluorescence intensity was very weak and the time to reach maximum emission was very long, but at higher temperature, the fluorescence developed more quickly. The fluorescence intensity reached the maximum after the system was heated in a boiling waterbath for 10 min.

Effect of 2-Cyanoacetamide Concentration

The effect of the concentration of 2-cyanoacetamide on the fluorescence intensity of the system is shown in Fig. 4. The result indicated that the fluorescence intensity of the system increased with increasing 2-cyanoacetamide concentration up to 0.2% (w/v), and then remained stable. A 0.3% (w/v) 2-cyanoacetamide was selected for the assay.

Effect of Adding Order of Reagents

Experiments showed that the addition order of the reagents may have an effect on the fluorescence intensity. On the basis of these results, we selected borate–NaOH buffer solution, 3,4-dihydroxyphenylalanine, and 2-cyanoacetamide solution as the best order for this assay.

In addition, it was indicated that when the system was heated in a boiling water bath for 10 min and cooled to room temperature, the fluorescence intensity of this system could reach a maximum immediately and remained stable for at least 7 hr.

Table I. Effect of Buffers (3,4-Dihydroxyphenylalanine: 5×10^{-7} mol/L)

Buffers	Borate–NaOH	Na_2HPO_4	NH_3–NH_4Cl	0.1 mol/L NaOH
If (%)	100	75	48	60

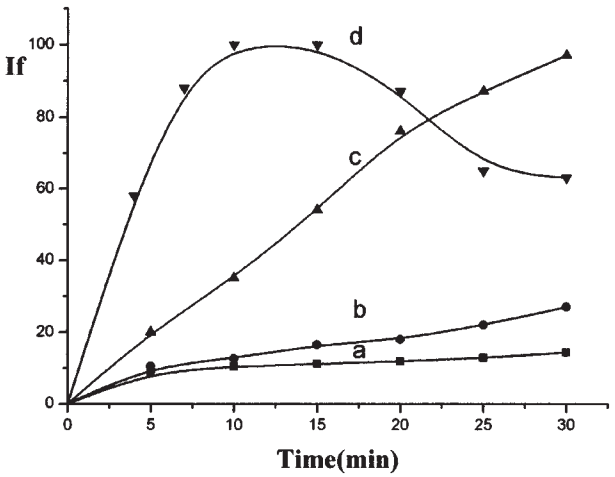


Fig. 3. Effects of temperature and heating time. (a) 25°C; (b) 40°C; (c) 60°C, and (d) 100°C. Conditions: L-dopa: 5×10^{-7} mol/L; borate–NaOH buffer: 1.0 mL (pH = 13.2); 2-cyanoacetamide solution 2% (w/v): 1.5 mL.

Effects of Other Amino Acids and Catecholamines

The effects of other amino acids and catecholamines on the fluorescence intensity of the system were also examined. At a 5×10^{-7} mol/L concentration of 3,4-dihydroxyphenylalanine, the highest permissible molar excesses of other amino acids and catecholamines (i.e., causing a $\leq$5% relative error in the fluorescence intensity) are given in Table II. From Table II, it can be seen that the most amino acids do not interfere or interfere slightly. E and NE do not interfere with the determination

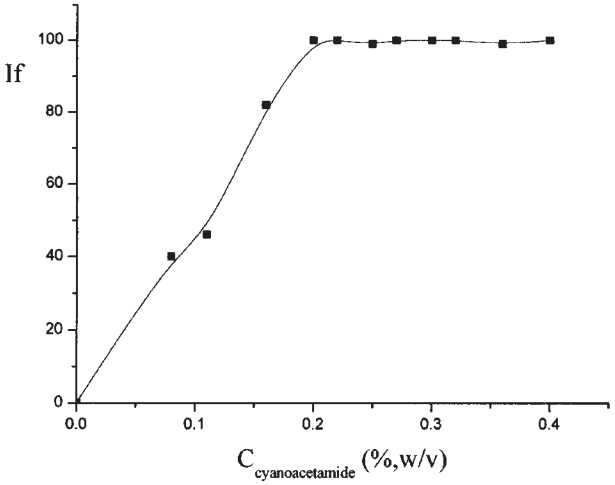


Fig. 4. Effect of 2-cyanoacetamide concentration. Conditions: 3,4-dihydroxyphenylalanine: 5×10^{-7} mol/L; borate–NaOH buffer: 1.0 mL (pH = 13.2).

Table II. Effects of other amino acids and catecholamines 3,4-dihydroxyphenylalanine: (5×10^{-7} mol/L)

Amino acids and catecholamine	Highest permissible molar excess	Relative error
L-Phe	12	3.7
DL-Thr	22	−1.9
L-Asp	6	4.6
L-His	10	−2.9
DL-Tyr	10	3.9
L-Ala	10	2.7
L-Gly	2	3.8
L-Tyr	3	4.9
L-Cys	4	1.6
HCL-Lys	10	2.4
L-Arg	10	3.5
Anthranilic acid	3	3.5
Uric acid	8	4.0
Valine	3	2.1
γ-Collidine	10	−2.5
Ammoniatriacetic acid	10	3.9
Epinephrine	10	1.8
Norepinephrine	20	3.3
HSA	1.7	−3.6
BSA	1.3	−4.2

of 3,4-dihydroxyphenylalanine. Thus, the selectivity of this method is better than that of the common fluorimetric methods reported [8–11], whereas the interferences from human serum albumin (HSA) and bovine serum albumin (BSA) could be resolved by using HPLC and sample preparation techniques [2].

ANALYTICAL APPLICATION

Standard Curve and Detection Limit

When 3,4-dihydroxyphenylalanine concentration was in the range of 2.5×10^{-8} to 2.0×10^{-6} mol/L, a linear relationship was observed between fluorescence intensity and the 3,4-dihydroxyphenylalanine concentration. The detection limit is 6.8×10^{-9} mol/L with an S/N of 3.

A comparison between the sensitivity of this method and other common spectrometric methods is summed up in Table III. The sensitivity of this method is higher than most other spectrometric methods. Although the sensitivity of this method is comparable to those of the paracetamol methods [11] and the method using Tb^{3+} as the fluorescence probe [10], it has additional advantages: the heating time of this method is 10 min, whereas that with paracetamol is 40 min, and the selectivity of this method is better than that of the method using Tb^{3+} as fluorescence probe.

Recovery Test

The standard addition method was applied in the recovery test. Two samples were determined and are listed in Table IV. Table IV, shows that the recoveries of 3,4-dihydroxyphenylalanine are 97.7% and 98.6%, respectively.

Sample Analysis

The present method was applied to the determination of L-dopa (made in Shanghai Fuda Medicine Company, Shanghai, China) and compared with the method using rare earth Tb^{3+} as a fluorescence probe [10]. Table V shows that the precision and accuracy of the proposed method are satisfactory.

Mechanism of the Reaction

2-Cyanoacetamide could be used for fluorimetric determination of reducing carbohydrates [13]. 3,4-Dihydroxyphenylalanine and reducing carbohydrates have tautometric enediol and α-hydroxycarbonyl groups, respectively. Thus, the fluorescence reactions of these two groups of compounds should be essentially of the same type, namely producing *N*-heterocyclic compounds by dehydrative cyclization of the condensates.

Table III. Comparison of Different Methods

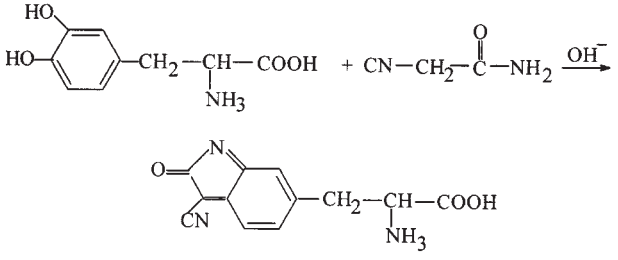
Methods	Probes or reagents	Detection limits (ng/mL)	Reference
Flow-injection chemiluminescence	Potassium permanganate	62	7
UV spectrophotometry	Cu (II)	1970	4
Flow-injection spectrophotometry	Sweet potato root as polylhenol oxidese	2958	5
Flow-injection spectrophotometry	*p*-Aminophenol	52	6
Fluorometry	Tb(III)	1.5	10
Fluorometry	Paracetamol	1.0	11
This work	2-cyanoacetamide	1.3	

Table IV. Recovery Test ($\times 10^{-7}$ mol/L)

Composition of mixtures	3,4-Dihydroxyphenylalanine found				Mean	Standard deviation	Recovery ratio (%)
3,4-Dihydroxyphehylalanine 3.0 DL-Thr 6.0 L-His 3.0 L-Asp 3.0 DL-Tyr 3.0	2.95	2.93	2.95	2.92 2.90	2.93	0.02	97.7
3,4-Dihydroxyphenylalanine 5.0 L-Phe 10.0 DL-Thr 5.0 L-His 5.0 L-Ala 10.0 L-Arg 6.0	4.92	4.91	4.95	4.94 4.93	4.93	0.02	98.6

Table V. Results of Sample Analysis ($\times 10^{-7}$ mol/L)

Proposed method			Method using Tb as fluorescence probe[8]		
Sample concentration	Mean	SD	Sample concentration	Mean	SD
2.02 1.97 2.02 1.95 1.95	1.98	0.032	2.01 1.95 2.02 2.03 1.97	1.99	0.038



Scheme 1

CONCLUSION

Experiments indicated that at pH = 13.0–13.3, 3,4-dihydroxyphenylalanine could react with 2-cyanoacetamide and emit strong fluorescence with the excitation and emission peaks of 335 and 405 nm. Its detection limit is 6.8×10^{-9} mol/L, and most amino acids, E, and NE do not interfere with the determination of 3,4-dihydroxyphenylalanine. The proposed method has been used for the determination of 3,4-dihydroxyphenylalanine in pharmaceutical preparations. In comparison with other common spectrometric methods reported, the proposed method possesses higher sensitivity and selectivity.

ACKNOWLEDGMENT

This work was supported by Natural Science Foundations of China and Shandong Province and by the Visiting Scholar Foundation of Key Lab in Shandong University, Jinan, China.

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