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A Sensitive Fluorimetric Method for the Determination of Epinephrine

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A sensitive fluorimetric method for the determination of epinephrine (E) is described in this paper. The experiments indicate that epinephrine can react with formaldehyde (HCHO) in an acid medium to form a condensation product, which can be oxidized by potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$) in borax buffer (pH = 9.5). The reaction product can emit strong fluorescence. Ascorbic acid (AA) is used in order to consume excess potassium hexacyanoferrate and stabilize the fluorescent product. Under optimum conditions, a linear relationship has been obtained between the fluorescence intensity and the concentration of epinephrine in the range of $1.4 \times 10^{-9} - 2.1 \times 10^{-6}$ mol/l, and the detection limit is 2.4×10^{-10} mol/l (4.3×10^{-11} g/ml, $S/N = 3$). The method is applied for the determination of E in both actual sample and the synthetic sample with E and norepinephrine (NE) by using the coupling technique of synchronous fluorimetry and H-point standard addition method, and the results obtained are satisfactory.

KEY WORDS: Epinephrine; formaldehyde; potassium hexacyanoferrate(III); fluorimetric analysis.

INTRODUCTION

Epinephrine (E) plays important roles as a neurotransmitter and a hormone. It can maintain the balance of homeostasis of the body. Thus, the determination of epinephrine has attracted much attention. Fluorimetric method and high-performance liquid chromatography method [1] are often used in the determination of epinephrine. Recently, others have also been researched, for example, electrochemical method [2–4], capillary electrophoresis method [5,6], and flow injection chemiluminescence method [7,8]. Several fluorimetric methods such as the trihydroxyindole (THI) method [9], terbium(III) fluorescence probe method [10] and condensation method [11,12] have been reported, but their detection limits are not low enough, and it is difficult to meet the actual needs in practice.

The proposed method is based on the condensation reaction of epinephrine with HCHO, and the condensation product is oxidized by $K_3[Fe(CN)_6]$ in borax buffer (pH 9.5) to form a fluorophore. Ascorbic acid (AA) is used in order to consume excess potassium hexacyanoferrate and stabilize the fluorescent product. Experiments indicate that the proposed method is sensitive. It is used in determining epinephrine in both injection and simultaneous determination of the synthetic mixed sample with E and NE by using the coupling technique of synchronous fluorimetry and H-point standard method with satisfactory results.

EXPERIMENTAL

Apparatus

All fluorescence measurements were made on a FL-4500 spectrofluorimeter (Hitachi, Japan). All pH

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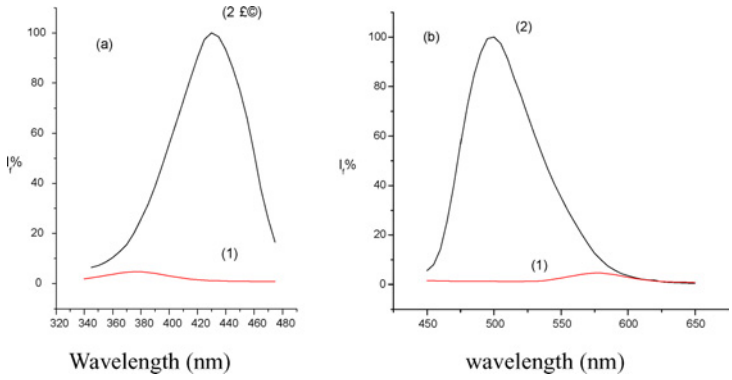


Fig. 1. Fluorescence spectra. (a) Excitation spectra ($\lambda_{em} = 498$ nm); (b) Emission spectra ($\lambda_{ex} = 428$ nm). (1) reagent blank; (2) E-HCHO -$K_3[Fe(CN)_6]$-AA- system. Conditions: E, 1.36×10^{-7} mol/l; HCHO, 0.2% (v/v); $Na_2B_4O_7$-NaOH, 0.01 mol/l (pH = 9.5); $K_3[Fe(CN)_6]$, 0.025% (w/v); AA, 0.012% (w/v). (b) O_7-NaOH, 0.01 mol/l (pH = 9.5); $K_3[Fe(CN)_6]$, 0.025% (w/v); AA, 0.012% (w/v).

measurements were made on a Delta 320-S pH meter (Mettler Toledo).

Regents and Solutions

An epinephrine standard stock solution (1.36×10^{-3} mol/l) was prepared by dissolving 0.0250 g epinephrine (Fluka, HPLC) in 100 ml of 0.01 mol/l hydrochloric acid and stored in a refrigerator at 4°C. Working solution was prepared by diluting with distilled water.

Formaldehyde solution (2%, v/v) was prepared by diluting 5.0 ml 40% formaldehyde to 100 ml with distilled water.

Borax buffer solution (0.1 mol/l) was prepared by dissolving 3.81 g borax in 100 ml with distilled water and adjusted pH to 9.5 with NaOH (6 mol/l).

Potassium hexacyanoferrate(III) solution(0.25%, w/v) was prepared by dissolving 0.250 g potassium hexacyanoferrate in 100 ml with distilled water.

Ascorbic acid solution (0.15%, w/v) was prepared by dissolving 0.150 g ascorbic acid in 100 ml with distilled water.

Experimental Procedure

First, an appropriate amount of epinephrine solution (0.01 mol/l hydrochloric acid) and 1 ml of formaldehyde solution (2%, v/v) is added, to a 25 ml test tube, and the solution is shaken and allowed to stand for 3 min in room temperature in order that they react perfectly in an acid medium. Second, 1 ml of borax buffer solution (0.1 mol/l, pH = 9.5) and 1 ml of potassium hexacyanoferrate solution (0.25%, w/v) are added, and the solution is shaken. Then, 0.8 ml of ascorbic acid solution (0.15%, w/v) is added. The tube is heated for 1.5–2.0 min in a boiling water bath and then cooled rapidly to room temperature. The volume is made up to 10 ml with distilled water. The fluorescence intensity of the system is measured in a 1 cm quartz cell with excitation and emission wavelengths of 428.0 nm and 498.0 nm, respectively. The slits of excitation and emission are 10 nm.

RESULTS AND DISCUSSION

Fluorescence Excitation and Emission Spectrum

Excitation and emission spectra of the E-HCHO-$K_3[Fe(CN)_6]$-AA system are shown in Fig. 1. From Fig. 1,

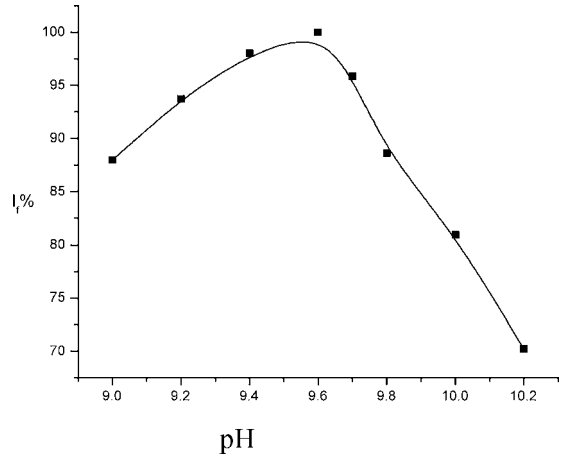


Fig. 2. Effect of pH on the fluorescence intensity. Conditions: E, 1.36×10^{-7} mol/l; HCHO, 0.2% (v/v); $K_3[Fe(CN)_6]$, 0.025% (w/v); AA, .012% (w/v).

Table I. Effect of Buffers Solution (pH 9.5 ± 0.1)

	$Na_2B_4O_7$-NaOH	HMA	Tris	NH_4Cl—NH_3
I_f (%)	100	1	1.8	1.5

it can be seen that the fluorescent intensity of the system is very strong. The excitation and emission peaks are 428 nm and 498 nm, respectively, and the reagent blank is low in this work, which benefits determining epinephrine.

Optimization of the General Procedure

Effect of pH and Buffers Solution

Figure 2 shows the effect of pH on the fluorescence intensity of the system. The maximum fluorescence intensity is obtained in the pH range of 9.4–9.6. Fixing pH = 9.5, at the effects of different kinds of buffers on the fluorescence intensity of the system are shown in Table I. The results indicate that 1.0 ml of $Na_2B_4O_7$-NaOH (0.1 mol/l, pH = 9.5) solution in 10 ml mixture is the most suitable buffer.

Effect of Formaldehyde Concentration

The effect of HCHO amount on the fluorescence intensity of the system is shown in Fig. 3(a). It is found

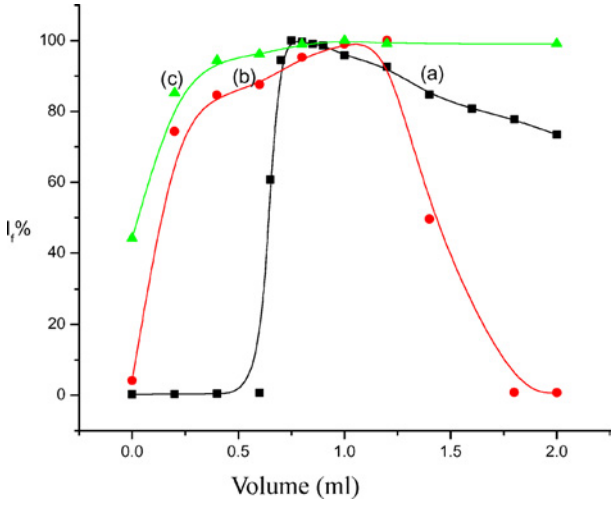


Fig. 3. (a) Effect of HCHO on the fluorescence intensity. Conditions: E, 1.36×10^{-7} mol/l; $Na_2B_4O_7$-NaOH, 0.01 mol/l (pH = 9.5); $K_3[Fe(CN)_6,]$, 0.025% (w/v); AA, 0.012% (w/v). (b) Effect of $K_3[Fe(CN)_6]$ on the fluorescence intensity. Conditions: E, 1.36×10^{-7} mol/l; HCHO, 0.2% (v/v); $Na_2B_4O_7$-NaOH, 0.01 mol/l (pH = 9.5); AA, 0.012% (w/v). (c) Effect of ascorbic acid on the fluorescence intensity. Conditions: E, 1.36×10^{-7} mol/l; HCHO, 0.2% (v/v); $Na_2B_4O_7$-NaOH, 0.01 mol/l (pH = 9.5); $K_3[Fe(CN)_6]$, 0.025% (w/v).

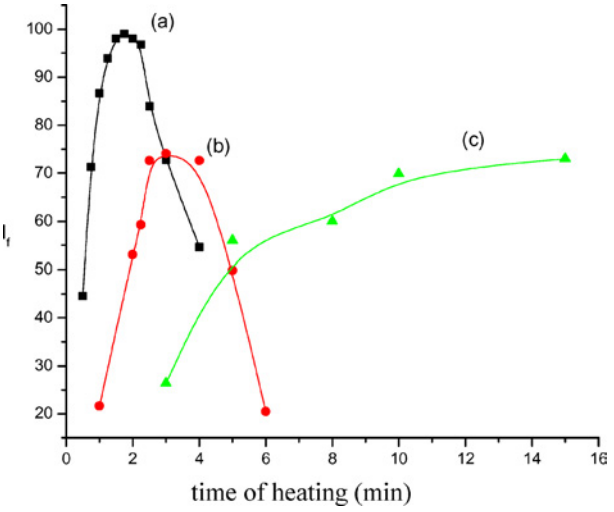


Fig. 4. Effect of oxidization temperature and time on the fluorescence intensity. (a) 100°C; (b) 80°C; (c) 60°C. Conditions: E, 1.36×10^{-7} mol/l; HCHO, 0.2% (v/v); $Na_2B_4O_7$-NaOH, 0.01 mol/l (pH 9.5); $K_3[Fe(CN)_6]$, 0.025% (w/v); AA, 0.012% (w/v).

that when the amount of HCHO is lower than 0.6 ml (0.15%, w/v), the fluorescence intensity is rapidly enhanced with the increase of HCHO concentration, then the fluorescence intensity reaches a maximum and remains stable in the range of 0.6–2.0 ml (2%, v/v) under the other conditions fixed. So 1 ml of HCHO solution (2%, v/v) in 10 ml mixture is selected in the work.

Table II. Interfere Test (1.36×10^{-7} mol/l E)

Foreign substance	The mole ratio between foreign substance and E	Relative error (%)
$AlCl_3$	19118	2.7
$Al_3(SO_4)_3$	5882	2.8
$BaCl_2$	1471	4.1
$CaCl_2$	2941	5.3
KCl	1838	4.8
$MgSO_4$	3309	4.9
$MnSO_4$	735	5.1
NaCl	2941	5.1
NH_4Cl	4412	4.1
$ZnCl_2$	1471	4.6
dopa	1000	7.8
NE	1	10
RNA	33	5.0
fsDNA	184	4.6
ctDNA	32	5.8
HSA	686	4.7
Glucose	2000	4.6
L-Tyrosine	29	5%
L-Typtophan	2206	4.4%

Table III. Comparison of Fluorimetric Methods for Determination of E

Method	Linear relationship (mol/l)	Detection limit (mol/l)	Reference
The proposed method	$1.4 \times 10^{-9} - 2.1 \times 10^{-6}$	2.4×10^{-10}	
Trihydroxyindole (THI) method	$4.1 \times 10^{-7} - 4.8 \times 10^{-5}$	2.7×10^{-8}	[9]
Terbium fluorescenceprobe method	$8.2 \times 10^{-8} - 9.8 \times 10^{-5}$	2.25×10^{-8}	[10]
2,3-Diaminonaphthalene	$6.0 \times 10^{-8} - 1.0 \times 10^{-5}$	5×10^{-8}	[11]
o-Phenylenediamine	$2.0 \times 10^{-8} - 6.0 \times 10^{-6}$	9.3×10^{-9}	[12]

Effect of Potassium Hexacyanoferrate (III) Concentration

The experiments indicate that the fluorescence intensity of the system is very weak in the absence of potassium hexacyanoferrate(III). Whereas the addition of $K_3[Fe(CN)_6]$ makes the fluorescence intensity of the system enhance considerably, the result is shown in Fig. 3(b). It can be seen that when the amount of $K_3[Fe(CN)_6]$ is lower than 1.4 ml (0.25%, w/v), the fluorescence intensity increases slowly with the increase of $K_3[Fe(CN)_6]$ concentration, then the fluorescence intensity reaches a maximum and remains stable in the range of 0.8–1.4 ml (0.25%, w/v); Whereas the amount of $K_3[Fe(CN)_6]$ is higher than 1.4 ml (0.25%, w/v), the fluorescence intensity has a swift decline with the increase of $K_3[Fe(CN)_6]$ concentration. Therefore, 1.0 ml of $K_3[Fe(CN)_6]$ solution (0.25%, w/v) is fixed in further experiments.

Effect of Ascorbic Acid Concentration

The effect of ascorbic acid amount on the fluorescence intensity of the system is shown in Fig. 3(c). It is found that when the amount of ascorbic acid is lower than 0.75 ml (0.15%, w/v), the fluorescence intensity increases promptly with the increase of ascorbic acid concentration, then the fluorescence intensity reaches a maximum and remains stable in the range of 0.75–0.95 ml (0.15%, w/v) under the other conditions fixed. For this reason, 0.8 ml of ascorbic acid solution (0.15%, w/v) in 10 ml mixture is the best in the work.

Effect of Oxidization Temperature and Time

The effects of oxidization temperature and time on the development of fluorescence are studied and the results are shown in Fig. 4. It can be seen that when the temperature is low, oxidization is slow and the degree of oxidization is low. At higher temperature, the fluorescence develops more rapidly and the degree of oxidization is higher. In our experiment, the heating time of 1.5–2.0 min in a boiling water bath is selected under the given conditions.

Effect of Foreign Substances

Under the optimum conditions, the effects of foreign substances including the dopa, norepinephrine (NE), ribonucleic acid (RNA), deoxyribonucleic acid (DNA), human serum albumin (HAS), glucose, some of amino acids, and familiar metal ions on the fluorescence intensity of the system are examined at 1.36×10^{-7} mol/l E. The relative errors in the fluorescence intensity are shown in Table II. The results suggest that most of the substances tested do not interfere or have little interference; however, norepinephrine (or dopamine) has obvious interference, which can be eliminated using the coupling technique of synchronous fluorimetry and H-point standard addition method [13]; larger amounts of RNA, ctDNA and L-tyrosine have some interfering, they should be eliminated. In addition, we consider that stronger reductants and amino compounds might have finite interfering because of consuming the reagents in the system. It is possible that some organic and biologic compounds with the fluorescence in the range of 470–570 nm interfere with the detection of E.

ANALYTICAL APPLICATION

Calibration Curve and Detect Limit

Under the optimum conditions defined here, a linear relationship is obtained between the fluorescence intensity

Table IV. Sample Recovery in Serum and Urine

Sample (10^{-1} μg/ml)	Found (10^{-1} μg/ml)	Mean $\pm$ SD	Recovery (%)
Urine 1.00	1.029, 0.998, 0.984, 0.943, 1.015	0.994 ± 0.033	99.4
Serum 1.00	0.953, 0.974, 1.012, 0.981, 0.985	0.981 ± 0.021	98.1

Note. Urine and serum were diluted 100-fold in the final assay solutions.

Table V. Sample Determination

The proposed method		THI method	
Found E ($\times 10^{-2}$ μg/ml)	Mean ± SD	Found E ($\times 10^{-2}$ μg/ml)	Mean ± SD
2.06, 2.10, 2.04, 2.04, 2.05	2.06 ± 0.025	2.06, 2.01, 2.02, 2.01, 1.99	2.02 ± 0.026

and the concentration of epinephrine in the range of $1.4 \times 10^{-9} - 2.1 \times 10^{-6}$ mol/l, with a detection limit of 2.4×10^{-10} mol/l ($S/N = 3$). The regression equation is as follows: $Y = 14.74835 + 3.19324 \times 10^{9}$ C, with a correlation coefficient of 0.9992. It can be found that the proposed method has a lower detection limit, compared with other fluorimetric methods is shown in Table III.

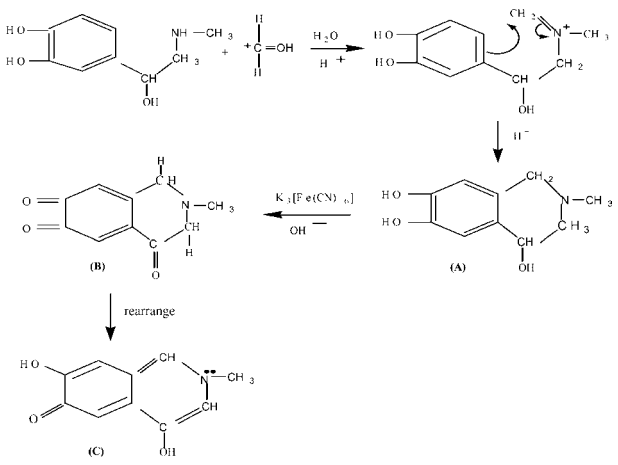
Sample Determination and Recovery Tests

Considering the effects of foreign substances on the fluorescence intensity of the system, the standard addition method is used for the recovery test and sample analysis. Tests of the recovery efficiency for known amounts of epinephrine in serum and urine were made. The results are shown in Table IV and indicate a 98–99% recovery. The proposed method is used for determining epinephrine in the epinephrine hydrochloride injection (Shanghai Harvest Pharmaceutical Co. Ltd), and compared with the original trihydroxyindole (THI) method. The results are shown in Table V. It can be seen that the results are satisfactory.

In order to eliminate the interfere of NE with the determination of E in the synthetic sample, the coupling technique of synchronous fluorimetry and H-point standard addition method [13] is used, and the result is shown in Table VI. It can be seen that the result is satisfactory.

MECHANISM OF THE FLUORESCENCE REACTION

Epinephrine can easily react with HCHO in an acid medium (0.005 mol/l hydrochloric acid) and can be oxidized by $K_3[Fe(CN)_6]$ in an alkali solution (borax buffer pH = 9.5), but the reaction mechanism is complex. In our experiment, according to the results of experiment and other reaction mechanism previously mentioned [11,12], the probable reaction mechanism for epinephrine is as follows:



First, epinephrine reacts with HCHO in an acid medium to form condensation product (A), and a molecule of water is dehydrated. Then, the condensation product is oxidized by $K_3[Fe(CN)_6]$ in borax buffer solution (pH = 9.5) to form the product (B), which rearranges to form 6-oxo-4,7-dihydroxy-2-methyl-*iso*-quinoline (ODHMIQ) (C), which can emit strong fluorescence. Ascorbic acid is used in order to consume excess potassium hexacyanoferrate and stabilize the fluorescent product.

Table VI. Simultaneous Determination of the Synthetic Sample with E and NE

	Sample (10^{-1} μg/ml)	Found (10^{-1} μg/ml)	Mean ± SD
E	1.00	1.007, 0.996, 1.078, 1.013	1.02 ± 0.037
NE	1.00	1.054, 0.996, 10.9, 1.005	1.01 ± 0.036

CONCLUSION

A sensitive fluorimetric method for the determination of epinephrine is proposed. The experiments indicate that epinephrine can react with formaldehyde in an acid medium to form condensation product, which can be oxidized by potassium hexacyanoferrate in borax buffer solution (pH = 9.5). The reaction product emits strong fluorescence. Ascorbic acid is used in order to consume excess potassium hexacyanoferrate and stabilize the fluorescent product. The excitation and emission peaks of the system are located at 428 nm and 498 nm, respectively. Under the

optimum conditions, the fluorescence intensity of the system is in proportion to the concentration of epinephrine in the range of $1.4 \times 10^{-9} - 2.1 \times 10^{-6}$ mol/l, the detection limit is 2.4×10^{-10} mol/l ($S/N = 3$), which is lower than that of other traditional fluorimetries for the determination of epinephrine. The proposed method has been used for the determination of epinephrine in both actual sample and the synthetic sample with E and NE by using the coupling technique of synchronous fluorimetry and H-point standard addition method. The reaction mechanism is also discussed.

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