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ORIGINAL PAPER

A New Spectrofluorimetric Method for Determination of Trace Amounts 5-Hydroxytryptamine in Human Urine and Serum

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Abstract A new spectrofluorimetric method was developed for the determination of trace amount of 5-hydroxytryptamine (5-HT) in human urine and serum samples. In the NaAc-HAc buffer solution of pH = 5.80, 5-HT can react with formaldehyde-acetylacetone system to form a new compound which sends yellow green fluorescence at 533nm and the enhanced fluorescence intensity is in proportion to the concentration of 5-HT. Optimum conditions for the determination of 5-HT were also investigated. The dynamic range and detection limit for the determination of 5-HT are $5.35 \times 10^{-7} \sim 1.07 \times 10^{-4}$ mol/L and 2.08×10^{-7} mol/L, respectively. The developed method is simple, practical and can be successfully applied to determination of 5-HT in human urine and serum samples. Moreover, the enhancement mechanisms of the fluorescence intensity in the 5-HT - formaldehyde-acetylacetone system have been also discussed.

Keywords 5-hydroxytryptamine (5-HT) · Acetylacetone · Formaldehyde · Spectrofluorimetry

Introduction

5-hydroxytryptamine (5-HT), one of the metabolites of the tryptophan, is well known as a neurotransmitter. The increased content of 5-HT in human physiological fluids have been recognized in the patients with carcinoid syndrome, migraine and schizophrenia [1]. It has been shown that in the ongoing inflammatory process of the appendix, blood serotonin level increases. Upon release, 5-hydroxytryptamine (5-HT) is rapidly metabolized in the liver by the monoamine oxidase (MAO) system to 5-hydroxyindoleacetic acid (5-HIAA) and, thereafter, is secreted in the urine. So measurement of the urine 5-HT may be a reliable marker of inflammation of the appendix. Therefore, it is important for clinical and pathological investigations to establish a precise, sensitive and simple method for the measurement of 5-HT in human body fluids.

Many methods for the determination of 5-HT include high-performance liquid chromatography (HPLC) [2–6], liquid chromatography [7–11], flow injection [12], capillary electrophoresis method [13, 14], gas chromatography [15, 16], electrochemical method [17], spectrofluorometry [18, 19].

In this paper, 5-HT can react with formaldehyde-acetylacetone system to form a new compound which sends yellow green fluorescence at 533 nm and the enhanced fluorescence intensity is in proportion to the concentration of 5-HT in the NaAc-HAc buffer solution of pH = 5.80, so a new spectrofluorimetric method with high sensitivity and selectivity for the determination of 5-HT is established. This method is simple and can be successfully applied to determination of 5-HT in human urine and serum samples with satisfactory results. The mechanism of fluorescence enhancement in the 5-HT-formaldehyde–acetylacetone system was also studied.

Materials and methods

Reagents

All chemicals used were of analytical-reagent or higher grade. Doubly distilled demineralized water was used for the preparation of all solutions and for all determinations.

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A stock 5-hydroxytryptamine (5-HT) (Alfa Aesar) solution was directly dissolved in doubly distilled demineralized water. The working standard solution(5.35×10^{-5} mol/L) was freshly prepared by appropriate dilution with doubly distilled demineralized water.

A working standard formaldehyde (3.7%, V/V) (Sinopharm Chemical Reagent Co., Ltd) solution was directly diluted 10 times from 37% solution using doubly distilled demineralized water.

A working standard acetylacetone (1.98%, V/V) (Tianjin Chemical Reagent Factory of China) solution was directly diluted 50 times from 99% solution using doubly distilled demineralized water.

All stocking solution and working solutions given above were stored at 0∼4°C.

A NaAC-HAc buffer solution (0.10 mol/L, pH = 5.80) was used for the system.

Apparatus

All fluorescence measurements were carried out on an RF-540 recording spectrofluorimeter (Shimadzu, Kyoto, Japan). All pH measurements were made with a pHs-3C digital pH meter (Shanghai Leici Device works, China).

General procedure

To 10mL color comparison tubes, solutions were added in the following order: 1.5 mL 5.35×10^{-5} mol/L 5-HT solution, 1.0 mL formaldehyde, 1.0 mL acetylacetone solution and 0.5 mL buffer solution. The mixture was heated 25 min in boiling water and then cooled in ice water; last the mixture was diluted to the mark with double distilled demineralized water. The fluorescence intensity was measured at λex/λem = 422 nm/533 nm. The enhanced fluorescence intensity of formaldehyde-acetylacetone by 5-HT was represented as $\Delta F = F - F_0$. Here F and F_0 are the fluorescence intensities of the systems with and without 5-HT, respectively.

Results and discussion

Spectral characteristics of fluorescence

Fluorescence emission spectrum of 1. 5-HT-acetylacetone-formaldehyde, 2. acetylacetone-formaldehyde are shown in Fig. 1. Comparing curve 1 with curve 2 in Fig. 1, after the addition of 5-HT into the acetylacetone-formaldehyde solution, the emission spectrum shift from 475 nm to 533 nm, the fluorescence intensity of the ternary system (F) is increased and the fluorescence intensity of the binary system (F_0) is decreased at 533 nm, which indicates that 5-HT can form a new ternary complex with the acetylacetone-formaldehyde complex.

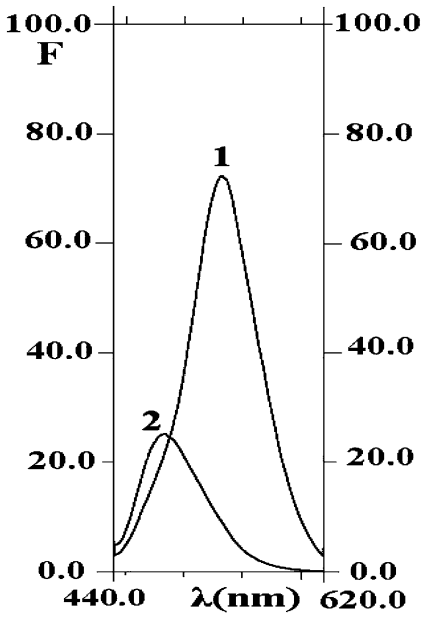


Fig. 1 Fluorescence emission spectrum Experimental condition: 3.0 mL 5.35×10^{-5} mol/L 5-HT solution, 1.0 mL acetylacetone, 1.0 mL formaldehyde, buffer pH = 5.80, λex/em = 422 nm/533 nm

Effect of experiment conditions

Effect of pH

The pH of the medium had some effect on the fluorescence intensity of the system, as shown in Fig. 2. The experimental results showed that the ΔF reached maximum at pH = 5.80. Therefore pH = 5.80 was selected with the using of 0.10 mol/L NaAC-HAc buffer solution for further study. As the volume of the buffer solution added from 0.3 mL to

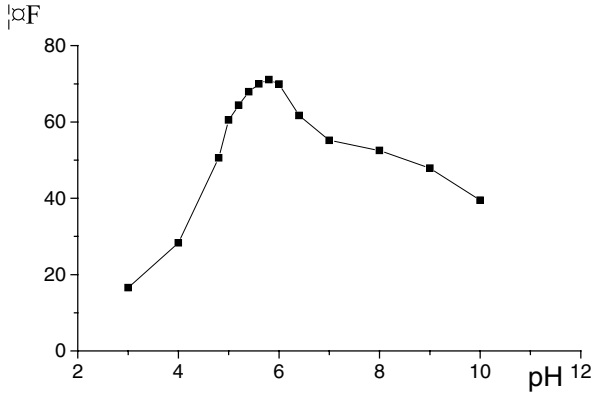


Fig. 2 The influence of pH on the fluorescence intensity of the 5-HT–formaldehyde–acetylacetone system The buffer solutions used were NaAc-HAc for pH = 3.0–6.0, KH_2PO_4 for pH = 7.0–8.0, Na_2CO_3-HCl for pH = 9.0–10.0

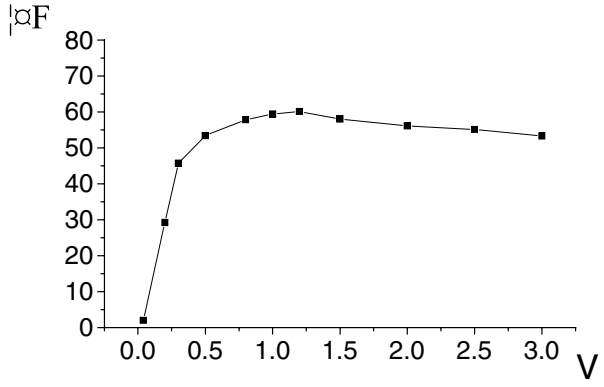


Fig. 3 The influence of formaldehyde concentration on ΔF Experimental condition: 8.03×10^{-6} mol/L 5-HT solution, formaldehyde (0.1 ~ 3.0 mL), 1.0 mL acetylacetone and 0.5 mL buffer solution

2 mL, the fluorescence intensity reached the maximum at 0.5 mL. So 0.5 mL was used for the following experiments.

Effect of formaldehyde concentration

Effects of formaldehyde concentration on the ΔF are shown in Fig. 3. In the system 5-HT-formaldehyde-acetylacetone, when formaldehyde in the range of 0.8 ~ 3.0 mL, the fluorescence intensity reached the maximum and remained constantly. So 1.0 mL was used for further study.

Effect of acetylacetone concentration

Effects of acetylacetone concentration on the proportion of ΔF are shown in Fig. 4. In the system 5-HT-formaldehyde-acetylacetone, when acetylacetone in the range of 0.8~1.2 mL, the proportion of ΔF reached the maximum and remained constantly. So 1.0 mL was used for further study.

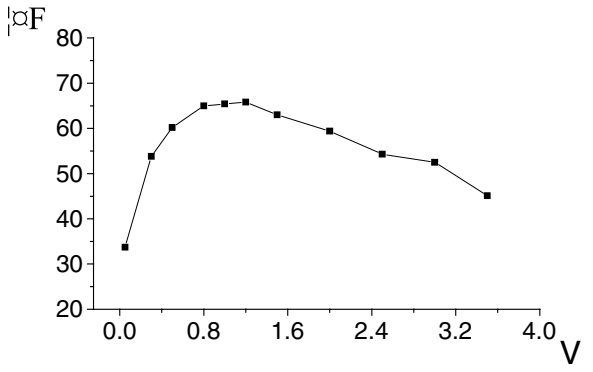


Fig. 4 The influence of acetylacetone concentration on ΔF Experimental condition: 8.03×10^{-6} mol/L 5-HT solution, 1.0 mL formaldehyde, acetylacetone (0.1~3.6 mL) and 0.5 mL buffer solution

Effect of the addition order of reagents

Adding the various reagents in different orders had influence on the F, F_0 and ΔF. The experimental results indicate that it was optimum when solutions were added in the following order: 5-HT, formaldehyde, acetylacetone and buffer. So 5-HT order was chosen in the following experiments.

Effect of reaction time

The chelation reaction was completed at about 10 min at room temperature. The fluorescence intensity reached its highest value and remained constantly for at least 180 min. Hence, all chelation reactions were carried out for 25 min at room temperature. All measurements were made at room temperature within 3 h.

Influence of coexisting substance

Series experiments of coexist substances had been carried out on the fluorescence of 5-HT-formaldehyde-acetylacetone system under the optimum condition. The concentrations of coexist substances approximate to that of human body fluid. Including mental ions, bases and some of the amino acid were also examined for interference; the experimental results are shown in Table 1. The criterion for interference is fixed at a ±10% variation of the average fluorescence intensity calculated for the established level of 5-HT, from Table 1 it can be seen that most coexisting substances are found to show no influence. Under the optimal experimental conditions for determination of 5-HT, histamine is found to show no influence also.

Analytical application

Linear range and limit of detection

Under the optimal experimental conditions, there was a good linear relationship between enhanced fluorescence value (ΔF) and concentration of 5-HT in the range of $5.35 \times 10^{-7} \sim 1.07 \times 10^{-4}$ mol/L with a correlation coefficient of 0.9958. The detection limit, as defined by IUPAC was determined to be 2.08×10^{-7} mol/L, when the K value was taken as 3 and the standard deviation was 0.0688 obtained from a series of 11 reagent blanks. And the precision of sample was 0.175 obtained from a series of 11 reagents.

Determination of 5-HT in human urine samples

The developed method was applied to the determination of 5-HT in human urine. Some of the amino acid, protein and many other substances in urine interfere seriously for the system, so we use extraction and re-extraction method according

Table 1 Influence of coexisting substances

Coexisting substances	Concentration (mol/L)	ΔF (%)	Coexisting substances	Concentration (mol/L)	ΔF (%)
Co^{2+}	2.50×10^{-7}	−2.13	L − cystine	2.00×10^{-7}	0.612
Ca^{2+}	2.00×10^{-6}	−2.07	Methionine	1.00×10^{-6}	1.73
Fe^{3+}	3.57×10^{-7}	−6.37	L-lysine	3.84×10^{-8}	−5.44
Mg^{2+}	5.00×10^{-5}	−4.30	L-tyrosine	5.00×10^{-8}	−9.02
Mo^{6+}	1.04×10^{-7}	2.81	Glycin	3.63×10^{-6}	−6.94
Cd^{2+}	1.00×10^{-7}	−0.735	Tryptophane	5.00×10^{-6}	−5.44
Cu^{2+}	5.00×10^{-5}	6.37	Lecithin	2.16×10^{-7}	1.02
Mn^{2+}	2.50×10^{-6}	−1.91	Glucose	2.20×10^{-4}	0.294
K^{+}	2.05×10^{-6}	−0.735	Uric acid	7.50×10^{-7}	−0.515
Na^{+}	5.00×10^{-3}	0.51	Bilirubin	5.00×10^{-7}	3.67
Adenine	3.55×10^{-7}	−1.86	Cholic acid	5.00×10^{-7}	3.23

Note. A 10.0 ml solution contained 5-HT (8.03×10^{-6} mol/l), 1.0 mL acetylacetone, 1.0 mL formaldehyde, 0.5 mL buffer solution and coexisting substances. $\Delta F\% = (F_1 - F_2)/F_2 \times 100\%$. Here, F_1 and F_2 are the fluorescence intensities of the systems with and without coexisting substances, respectively.

reference [20] to improve the selectivity of our method. Twenty-four-hour urine collections were obtained from two healthy volunteers (age 24 years). 10 mL urine was added to a 50 mL tube, then added 2.5 mL 1.0 mol/L hydrochloric acid and 1 mL 2% $FeCl_3$, mixed, stand for 10 min and filtrated to remove phenols. The filtrate was collected to another 50 mL tube which contained 4 g NaCl and 1 mL 2.5 mol/L sodium hydroxide, then 3 mL *n*-butanol was added, mixed, centrifuged and the *n*-butanol layer is separated from the aqueous layer. Another 2 mL *n*-butanol was added to the aqueous layer which was referred above, extracted again. The twice *n*-butanol layer (about 5 mL) was mixed and added to a separatory funnel which contained 5 mL *n*-heptane and 2.5 mL 1.0 mol/L hydrochloric acid, oscillated 10 min, and stand for 10 min, the 5-HT contained in urine sample was re-extracted into the layer of the hydrochloric acid, then the hydrochloric acid layer is separated from the butanol-heptane layer. At last the solution of hydrochloric acid layer was adjusted to pH = 7 with the solution of sodium hydroxide and analyzed by the developed method, using the standard compare method. The experimental result is shown in Table 2. The result was according with the report of the reference [2].

We added known amounts of 5-HT to urine that the concentration of 5-HT was known and determined the recovery of the 5-HT from the urine. The sample was treated as above-mentioned method, but as the increasing of content of 5-HT, the addition of reagents are increased, so 10 mL and 5 mL *n*-butanol was added instead of 3 mL and 2 mL *n*-butanol which was referred above, respectively. 15 mL *n*-heptane was added instead of 5 mL which was referred above. At last, the volume of the hydrochloric acid was 6 mL. From Table 2 it can be seen that the developed method can be easily performed and affords good precision and accuracy when applied to urine samples.

Determination of 5-HT in serum samples

The developed method was applied to the determination of 5-HT in human serum samples. Proteins in human serum interfere for the system. So 2 mL acetonitrile was added to 1.0 mL serum and centrifuged for 15 min at 4000 r/min to remove proteins. Then the samples were diluted 100 times and added known amounts of 5-HT to serum, and analyzed by a standard addition method. The experimental result is shown in Table 3. From Table 3 it can be seen that the developed method can be easily performed and affords good precision and accuracy when applied to human serum samples.

Table 2 Determination of 5-HT in urine samples

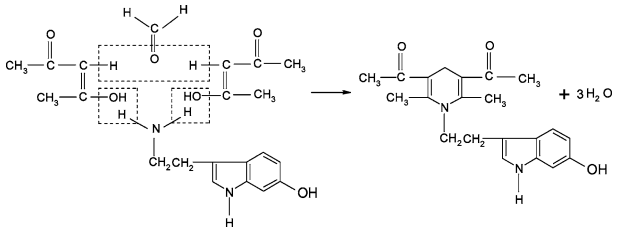
Urine samples	Original concentration (10^{-6}mol/L)	RSD %	Added (10^{-6}mol/L)	Total found (10^{-6}mol/L)	RSD %	Recovery %	Average Recovery%
	2.82		4.06	7.00		103.0	
Sample1	2.61	3.86	4.06	6.58	4.06	97.8	98.6
	2.72		4.06	6.58		95.1	
	7.53		6.38	13.82		98.6	
	7.63		6.38	13.76		96.1	
Sample2	7.45	1.19	6.38	14.07	4.02	103.8	99.5

Tables 3 Determination of 5-HT in serum samples

Serum samples	Added (10^{-6} mol/L)	Found (10^{-6} mol/L)	Average (10^{-6}mol/L)	Average recovery (%)	RSD %
1	5.80	6.08, 6.08, 6.00	6.05	104.3	0.76
2	9.28	9.36, 9.20, 9.44	9.33	100.6	1.31
3	11.6	11.4, 11.3, 11.5	11.4	97.6	0.88

Conclusion

A new spectrofluorimetric method was developed for determination of trace amount of 5-HT. According Hantzsch reaction, 5-HT can react with acetylacetone-formaldehyde to form a new ternary complex and can remarkably enhance the fluorescence intensity of the acetylacetone-formaldehyde complex at $\lambda = 533$ nm. The enhanced fluorescence intensity is in proportion to the concentration of 5-HT. The method has been successfully applied to the determination of 5-HT in human urine and serum samples.



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