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Fluorometric Determination of Biogenic Indole Compounds using Perchloric Acid

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The fluorescence properties of biogenic indole compounds in perchloric acid (PCA) solution were investigated. The indoles gave specific yellow-greenish fluorescence in PCA solution ($\lambda_{ex}=425$ nm and $\lambda_{em}=525$ nm for 3-substituted indoles; $\lambda_{ex}=300$ nm and $\lambda_{em}=525$ nm for 3,5-substituted indoles) under the optimal conditions. Based on the results of optimization studies for the fluorogenic reaction, two fluorometric methods for the determination of these substituted indoles at the 100 pmol level were developed.

Keywords——fluorescence; fluorometric determination; indoles; perchloric acid; L-tryptophan; 5-hydroxy-L-tryptophan; tryptophan metabolites

Many quantitative analyses have been developed for biogenic indole compounds due to their physiological importance. For example, L-tryptophan has been assayed fluorometrically by measuring either its native fluorescence[1,2] or that of the β-carboline induced by formaldehyde in the presence of an oxidizing reagent such as ferric chloride.[3,4] Serotonin (5-hydroxytryptamine) has been determined by measuring its fluorescence in 3 N hydrochloric acid[5–10] or by measuring the fluorescence of the reaction product with ninhydrin[11] or *o*-phthalaldehyde.[12] Thus, many methods for analyses of individual indoles have been reported. However, there has been no specific method for the detection of general biogenic indoles.

Previously, Tauber reported that L-tryptophan and some peptides containing L-tryptophan fluoresce in perchloric acid (PCA).[13,14] Nakamura and Pisano also found that 3-substituted indoles gave a specific yellow-orange fluorescence on silica gel plates upon being sprayed with 70% PCA.[15] As all biogenic indoles so far known in mammals are 3-substituted, this reaction seemed to be useful for a specific determination of the biogenic indoles. In the present investigation, therefore, we examined the optimum reaction conditions in PCA solution in order to develop specific methods for the fluorometric determination of these indoles using PCA.

Experimental

Materials——L-Tryptophan, indole-3-acetic acid, L-kynurenine, L-norepinephrine, cholic acid, deoxycholic acid, indole, 2-methylindole, 3-methylindole, indoleacetone, 5-methylindole, 5-nitroindole and 7-methylindole were purchased from Nakarai Chemicals (Kyoto). Tryptamine hydrochloride, 5-hydroxy-L-tryptophan, 5-hydroxytryptamine hydrochloride, 5-hydroxyindole-3-acetic acid, 5-methoxytryptamine hydrochloride, 5-methoxyindole-3-acetic acid, 3-hydroxy-DL-kynurenine, 3-hydroxyanthranilic acid, L-dopa, dopamine, 5-hydroxyindole, 6-hydroxymelatonin, DL-α-methyltryptophan, DL-4-fluorotryptophan, 6-methyl-DL-tryptophan, DL-6-fluorotryptophan, 6-fluorotryptamine, 7-methyl-DL-tryptophan and 7-methyltryptamine were purchased from Sigma Chemical Corp. (St. Louis, MO., U.S.A.); 5-methoxy-DL-tryptophan from Seikagaku Kogyo Co., Ltd. (Tokyo); L-epinephrine was from E. Merck (Darmstadt, G.F.R.); cholesterol from Kanto Chemical Co. (Tokyo); lithocholic acid, indole-β-carboxylic acid and indole-3-aldehyde from Tokyo Kasei Kogyo Co. (Tokyo); *N*-formyl-L-kynurenine from Calbiochem (La Jolla, Calif., U.S.A.); indole-3-acetonitrile from Wako Pure Chemical Industries Ltd. (Tokyo); 5-chloroindole and 1,2-dimethylindole from Aldrich Chemical Co. (Milwaukee, Wisc., U.S.A.). Chenodeoxycholic acid was a gift from Tokyo Tanabe Co., Ltd. (Tokyo). All compounds tested were dissolved in 0.2 M borate buffer (pH 8.5). Perchloric acid (70%, w/v) was purchased from Kanto Chemical Co. (Tokyo).

All other chemicals used were commercial guaranteed reagents.

Apparatus——A Hitachi MPF-2A fluorescence spectrophotometer was used. The spectra are uncorrected, so that the wavelengths in tables are tentative.

Procedure for the Fluorometric Determination of Indoles——In the following experiments, L-tryptophan, tryptamine, indole-3-acetic acid, 5-hydroxy-L-tryptophan, 5-hydroxytryptamine (serotonin), 5-hydroxyindole-3-acetic acid, 5-methoxy-DL-tryptophan, 5-methoxytryptamine and 5-methoxyindole-3-acetic acid were used as representative biogenic indoles. The fluorescence measurement was performed by the following procedures:

Procedure A for 3-Substituted Indoles: To 50 μl of a sample solution was added 3 ml of 55% (w/v) PCA. The mixture was allowed to stand at room temperature for 30 min and the fluorescence was measured at $\lambda_{ex}=425$ nm and $\lambda_{em}=525$ nm.

Procedure B for 3,5-Substituted Indoles: To 50 μl of a sample solution was added 3 ml of 30% PCA. The mixture was allowed to stand at room temperature for 30 min and the fluorescence was measured at $\lambda_{ex}=300$ nm and $\lambda_{em}=525$ nm.

Results

Excitation and Emission Spectra of Indoles in PCA Solution

In PCA solution, the excitation and emission maxima of the spectra of L-tryptophan both shifted to longer wavelengths as compared to those of the native fluorescence. On the other hand, the excitation maximum of 5-hydroxy-L-tryptophan in PCA was similar to that of the native fluorescence, while the emission maximum was shifted to longer wavelength (Fig. 1). Fluorescence characteristics of tryptamine and indole-3-acetic acid were similar to those of L-tryptophan, while the characteristics of 5-hydroxy- or 5-methoxyindoles tested were similar to those of 5-hydroxy-L-tryptophan, as shown in Table I.

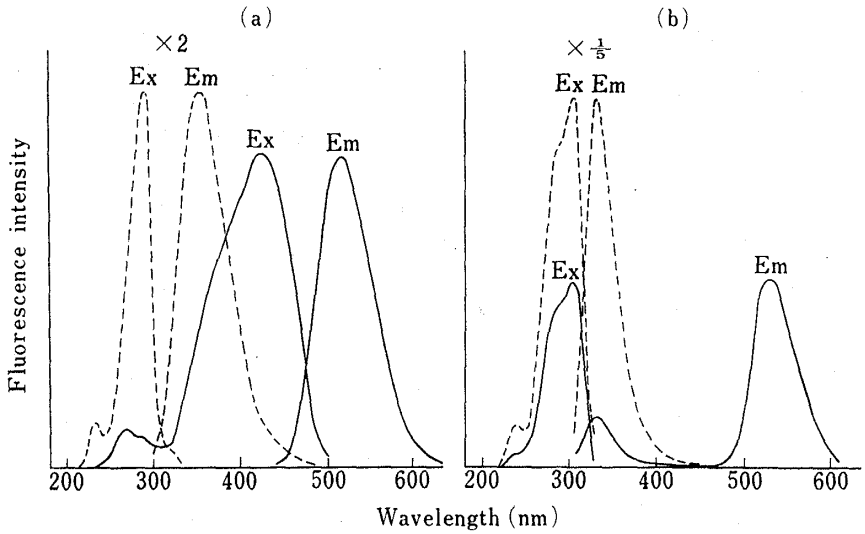

Fig. 1. Excitation and Emission Spectra of L-Tryptophan (a) and 5-Hydroxy-L-tryptophan (b)

Fifty μl of 10^{-4} M solution of a compound was mixed with 3 ml of PCA solution or phosphate buffer (PB). The mixture was allowed to stand at room temperature for 30 min, and the fluorescence was measured.
(a) ——: in 60% PCA, ----: in 0.5 M PB (pH 7.0); (b) ——: in 30%PCA, ----: in 0.5 M PB (pH 7.0).

Fluorescence of Indoles in Various Acid Solutions

Table II summarizes the characteristics of the fluorescence derived from L-tryptophan and 5-hydroxy-L-tryptophan by treatment with various acids. In the case of L-tryptophan, PCA and sulfuric acid induced fluorescence, though the fluorescence intensity obtained with the latter was only a fraction of that obtained with the former. In the case of 5-hydroxy-L-tryptophan, in addition to PCA and sulfuric acid, hydrochloric acid, phosphoric acid and trifluoromethanesulfonic acid also gave similar fluorescence. From the above results,

Table I. Excitation and Emission Maxima of Native and PCA-induced Fluorescence and Relative Fluorescence Intensities of Indole Compounds

Compound	Native[a]		PCA-induced[b]		
	λ_{ex} (nm)	λ_{em} (nm)	λ_{ex} (nm)	λ_{em} (nm)	RFI[c]
3-Substituted indoles:					
L-Tryptophan	288	355	426	520	100[d]
Tryptamine	289	353	442	525	76
Indole-3-acetic acid	291	360	423	524	14
3,5-Substituted indoles:					
5-Hydroxy-L-tryptophan	305	355	302	530	11
5-Hydroxytryptamine	304	333	302	534	8
5-Hydroxyindole-3-acetic acid	305	343	302	533	8
5-Methoxy-L-tryptophan	301	336	301	528	15
5-Methoxytryptamine	301	338	300	529	12
5-Methoxyindole-3-acetic acid	300	353	300	528	9

a) Measured in 0.5 M phosphate buffer (pH 7.0).
b) Measured in 55% PCA for 3-substituted indoles and 30% PCA for 3,5-substituted indoles.
c) Fluorescence measurement: Ex 425 nm, Em 525 nm for the fluorescence induced by 3-substituted indoles; Ex 300 nm, Em 525 nm for the fluorescence induced by 3,5-substituted indoles.
d) L-Tryptophan is arbitrarily taken as 100.

Table II. Fluorescence Properties of L-Tryptophan and 5-Hydroxy-L-tryptophan in Various Acid Solutions[a]

Acid	L-Tryptophan				5-Hydroxy-L-tryptophan			
	Optimal Conc. (%, w/v)	λ_{ex} (nm)	λ_{em} (nm)	RFI	Optimal Conc. (%, w/v)	λ_{ex} (nm)	λ_{em} (nm)	RFI
$HClO_4$	65	426	520	100[b]	30	302	530	100[b]
H_2SO_4	64	429	512	5	32	305	532	87
HCl		—[c]	—[c]	0	28	305	531	79
H_3PO_4		—	—	0	65	303	529	69
CH_3COOH		—	—	0		—	—	0
CF_3SO_3H		—	—	0	45	305	530	94

a) Fifty μl of 0.1 mM solution of a compound was mixed with 3 ml of an acid solution, and the fluorescence was measured after the mixture had been allowed to stand at room temperature for 30 min.
b) $HClO_4$ is arbitrarily taken as 100.
c) No fluorescence.

we decided to use PCA since both 3-substituted indoles and 3,5-substituted indoles gave the strongest fluorescence intensity with this acid.

Optimal Concentration of PCA

Fig. 2 shows the relationship between PCA concentration and fluorescence intensity. L-Tryptophan gave maximal fluorescence intensity in 65% PCA, and tryptamine and indole-3-acetic acid in 55% PCA. 3,5-Substituted indoles showed the maximal fluorescence intensity at around 30% PCA concentration.

Time Course

The relationship between reaction time and fluorescence intensity was investigated at room temperature (30°C) with 55% PCA for 3-substituted indoles and with 30% PCA for 3,5-substituted indoles. As shown in Fig. 3, the fluorescence intensities of L-tryptophan, tryptamine and indole-3-acetic acid reached the maxima after 15 min, 40 min and 20 min, respectively, and then decreased. On the other hand, 3,5-substituted indoles gave the highest

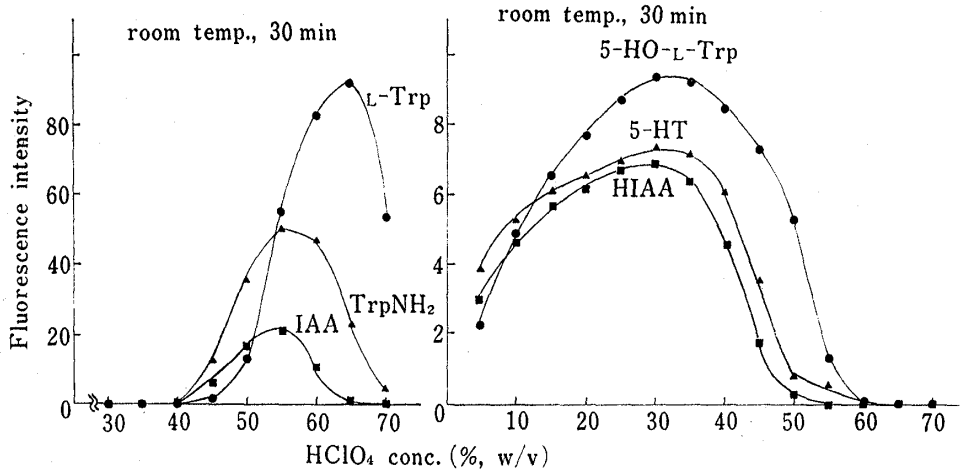

Fig. 2. Relationship between $HClO_4$ Concentration and Fluorescence Intensity

Abbreviations used are: L-Trp, L-tryptophan; $TrpNH_2$, tryptamine; IAA, indole-3-acetic acid; 5-HO-L-Trp, 5-hydroxy-L-tryptophan; 5-HT, 5-hydroxytryptamine; HIAA, 5-hydroxyindole-3-acetic acid. Fifty μl of 10^{-4} M solution of an indole was mixed with 3 ml of each concentration (w/v) of PCA, and the mixture was allowed to stand at room temperature for 30 min.

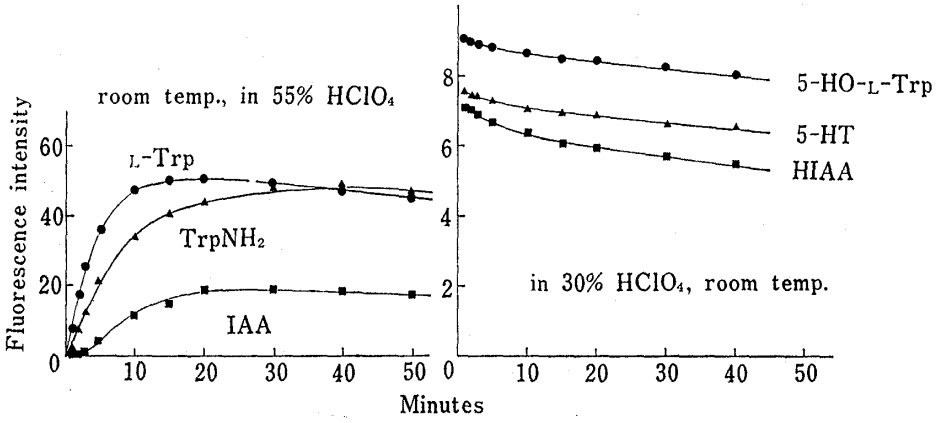

Fig. 3. Time Courses of the PCA-induced Fluorescence at 30°C

Fifty μl of 10^{-4} M solution of a compound was mixed with 3 ml of 55% PCA solution for 3-substituted indoles (left) or of 30% PCA solution for 3,5-substituted indoles (right), and then the fluorescence was measured after appropriate intervals.

fluorescence intensities immediately after adding PCA, followed by gradual decrease. To increase the precision of the determination, the fluorescence of 3,5-substituted indoles was measured in Procedure B 30 min after mixing, when the rate of the decrease of fluorescence intensity was smaller than the initial rate.

Effect of Temperature on the PCA Reaction

The effect of PCA concentration on the fluorescence reaction was examined at 75°C by using the same conditions as in Fig. 2 except for the temperature. Compared to the reaction at room temperature, lower fluorescence intensities were obtained with 3-substituted indoles. The fluorescence intensity at elevated temperature rapidly reached the maximum, followed by a rather distinct decrease (Fig. 4). On the other hand, the heat treatment did not affect either the fluorescence intensity or the reaction rate of 3,5-substituted indoles.

Determination of Indole Compounds

The working curves for L-tryptophan, tryptamine, 5-hydroxy-L-tryptophan and 5-hydroxytryptamine are shown in Fig. 5. The determination limit was 100 pmol for the above four compounds. The detection limit at which the fluorescence intensity was twice

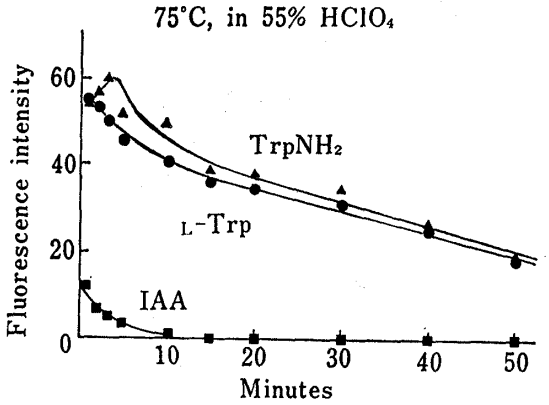

Fig. 4. Time Courses of the PCA-induced Fluorescence at 75°C

Conditions were the same as in Fig. 3 except for the reaction temperature.

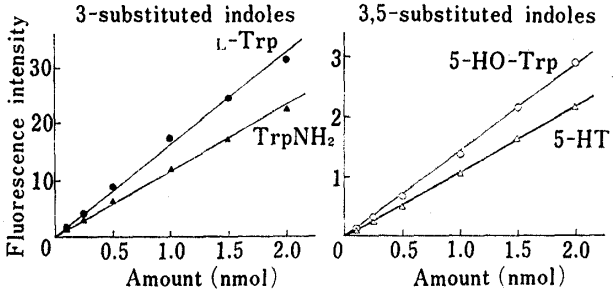

Fig. 5. Working Curves for Indole Compounds

that of the blank was 70 pmol for L-tryptophan and tryptamine, 30 pmol for 5-hydroxy-L-tryptophan and 50 pmol for 5-hydroxytryptamine. The reproducibility of the method was determined by assaying 500 pmol of compounds. The standard deviations of the measurements ($n=5$) were 1% for L-tryptophan and tryptamine, 3% for 5-hydroxy-L-tryptophan and 4% for 5-hydroxytryptamine.

With regard to the specificity of the present fluorometric determination (Table III), the members of the so-called "kynurenine pathway" such as *N*-formyl-L-kynurenine, L-kynurenine, 3-hydroxy-L-kynurenine and 3-hydroxyanthranilic acid yielded no fluorescence. L-Dopa and catecholamines, which have native fluorescence, did not interfere with this fluorometric determination. Steroids and bile acids which were detected on thin layer plates by spraying PCA reagent[16,17] yielded scarcely any fluorescence in the present procedure.

Table IV shows the relative fluorescence intensities of various substituted indoles obtained by the present fluorometric methods. Under the conditions of Procedure A, indoles not substituted at the 3-position yielded no fluorescence. Among 3-substituted indoles, however,

TABLE III. Specificity of the Methods

Compound	Relative fluorescence intensity in	
	55% $HClO_4$ (Ex 425, Em 525)	30% $HClO_4$ (Ex 300, Em 525)
L-Tryptophan	100[a]	
5-Hydroxy-L-tryptophan		100[b]
N-Formyl-L-kynurenine	1	3
L-Kynurenine	0	0
3-Hydroxy-L-kynurenine	0	0
3-Hydroxyanthranilic acid	0	1
L-Dopa	0	0
Dopamine	0	0
L-Norepinephrine	0	0
L-Epinephrine	0	0
Cholesterol	1	1
Cholic acid	1	0
Deoxycholic acid	1	0
Chenodeoxycholic acid	1	0
Lithocholic acid	0	0

a) L-Tryptophan is arbitrarily taken as 100.
b) 5-Hydroxy-L-tryptophan is arbitrarily taken as 100.

Table IV. Fluorescence of Various Indole Compounds in PCA Solution

Substitution in indole ring	Compound	Relative fluorescence intensity in 55% PCA[a] 425 nm/525 nm	Relative fluorescence intensity in 30% PCA[b] 300 nm/525 nm
None	Indole	0	0
C-2	2-Methylindole	0	0
C-3	L-Tryptophan	100	0
	3-Methylindole	4	1
	Indole-β-carboxylic acid	0	0
	Indole-3-aldehyde	0	0
	Indoleacetone	1	0
	Indole-3-acetonitrile	15	0
C-5	5-Methylindole	0	0
	5-Chloroindole	0	0
	5-Nitroindole	0	0
	5-Hydroxyindole	0	18
C-7	7-Methylindole	0	1
N-1, C-2	1,2-dimethylindole	0	0
N-1, C-3	DL-α-Methyltryptophan	10	0
C-3, C-4	DL-4-Fluorotryptophan	13	0
C-3, C-5	5-Hydroxy-L-tryptophan	1	100
C-3, C-6	6-Methyl-DL-tryptophan	0	0
	DL-6-Fluorotryptophan	0	0
	6-Fluorotryptamine	3	0
C-3, C-7	7-Methyl-DL-tryptophan	1	1
	7-Methyltryptamine	3	1
C-3, C-5, C-6	6-Hydroxymelatonin	0	95

a) Fifty μl of 10^{-4} M solution of indole was mixed with 3 ml of 55% PCA, and the fluorescence was measured after the mixture had been allowed to stand at room temperature for 30 min.
b) Fifty μl of 10^{-3} M solution of indole was mixed with 3 ml of 30% PCA, and the fluorescence was measured after the mixture had been allowed to stand at room temperature for 30 min.

indole-β-carboxylic acid and indole-3-aldehyde did not fluoresce. On the other hand, 5-hydroxyindole, 5-hydroxy-L-tryptophan and 6-hydroxymelatonin fluoresced under the conditions of Procedure B, while other compounds tested yielded little or no fluorescence. In the latter case, the substitution of a hydroxy or methoxy group at the 5-position is essential for the induction of the fluorescence, but substitution at the 3-position is not always necessary.

Thin Layer Chromatography of Fluorophores derived from 3-Substituted Indoles

Twenty milligrams of L-tryptophan was dissolved in 20 ml of 65% PCA. The reaction mixture was allowed to stand at room temperature for one hour, then neutralized with 10 M KOH. The supernatant was applied to a silica gel 60 thin layer chromatographic plate (E. Merck) and developed with benzene–ethanol (1 : 1). After air-drying of the plate, the fluorescence was observed under long-wavelength ultraviolet light. An unknown spot (Rf=0.42) which fluoresced blue was detected. When the plate was sprayed with 70% PCA, L-tryptophan (Rf=0.12) and the unknown product (Rf=0.42) gave yellow-greenish fluorescence. Similar results were obtained with tryptamine.

Discussion

It is known that indole compounds fluoresce in strongly acidic solutions. L-Tryptophan and some peptides containing L-tryptophan give yellow-greenish fluorescence in trifluoroacetic acid[18] as well as PCA.[13,14] However, the fluorescent species involved in the reactions have not been investigated precisely. With regard to the fluorophores derived from 3-substituted indoles, the following results obtained in this investigation strongly suggest the formation

of unknown fluorescent product(s); (i) the formation of fluorophores required a significant reaction time (15—40 min) (Fig. 3) and was accelerated by heating (Fig. 4), and (ii) the reaction mixture of 3-substituted indoles with PCA contained fluorescent compounds distinguishable from the parent compounds, as determined by thin layer chromatography. On the other hand, it is also well known that 3,5-substituted indoles such as serotonin fluoresce in hydrochloric acid solution.[19,20] As already shown in Table II, the spectral characteristics of the fluorescence derived from 3,5-substituted indoles in PCA were similar to those in hydrochloric acid. Moreover, the spectral change observed on going from PCA to neutral solution was reversible, probably through protonation, as was observed in hydrochloric acid.[21] Therefore, it could be considered that the fluorophore(s) in PCA might be the same as that in hydrochloric acid. Chen supposed that the fluorescent species formed from serotonin in hydrochloric acid might be its protonated excited-state form.[22] The facts that the induction of fluorophore(s) was fast (Fig. 3) and independent of heating seem to support his hypothesis.

Recently, many high performance liquid chromatographic (HPLC) methods have been developed for the analysis of biogenic indole compounds by utilizing ultraviolet absorption,[23] native fluorescence[23–27] and the electrochemical properties.[28,29] However, these detection methods are not highly specific and the determinations are often disturbed by other substances present in samples unless a suitable cleanup procedure is performed. Since the present fluorometric determination using PCA is specific for indole compounds, this fluorogenic reaction may be applied as a detection system in HPLC of biogenic indole compounds.

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