Fluorometric Determination of L-Tryptophan with Methoxyacetaldehyde¹⁾

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It was observed in our laboratory that methoxyacetaldehyde induced strong fluorescence with L-tryptophan in the presence of NaNO₂ at pH 2.75. The reaction mixture was heated at 80 °C for 15 min and the fluorescence was measured at the excitation wavelength, 253 nm and the emission, 450 nm. Amino acids other than L-tryptophan did not produce fluorescence under the conditions employed. A good linear working curve was observed between 25 pmol/50 μ l and 500 pmol/50 μ l of L-tryptophan. The limit of determination for L-tryptophan was 10 pmol/50 μ l. The coefficient of variation of the measurements ($n = 5$) was 1.5% for 250 pmol/50 μ l of L-tryptophan. The proposed method was successfully applied to the analyses of tryptophan in a pooled human serum. Total tryptophan was determined after deproteinization with trichloroacetic acid, and free tryptophan was determined using the ultrafiltrate of a pooled human serum. Analytical recoveries of total and free tryptophan were 98.8% and 99.1%, respectively. The fluorescent products of L-tryptophan and methoxyacetaldehyde were characterized as β -carboline and 1-methoxymethyl- β -carboline by the use of liquid chromatography-mass spectrometry (LC-MS) and HPLC.

Keywords L-tryptophan; serum; fluorometry; methoxyacetaldehyde; β -carboline; 1-methoxymethyl- β -carboline

L-Tryptophan (Trp) is the only amino acid that is found to be bound to albumin in human sera, and thus it occurs in two forms, free and bound to albumin.²⁾ It has been reported that the concentration of free tryptophan in the sera of autistic children and depressive patients with psychiatric diseases^{3–6)} vary significantly. As tryptophan is metabolized mainly in the liver, the concentration of free tryptophan in plasma is also taken into consideration as a measure in hepatic failure.^{7–9)} Moreover, it has been suggested that urinary tryptophan and its metabolites have something to do with bladder cancer.¹⁰⁾ In light of these facts, it is of clinical significance to determine the concentration of total tryptophan and free tryptophan in the body fluids.

Spectrofluorometric methods for the determination of tryptophan are divided into two groups; one utilizes its native fluorescence¹¹⁾ and the other the fluorescence of β -carbolines induced by Pictet-Spengler reaction between tryptophan and aldehydes such as formaldehyde,¹²⁾ chloroacetaldehyde (CAA)¹³⁾ and phenylglyoxal¹⁴⁾ under acidic conditions. Further studies on α -substituted acetaldehydes such as dichloro-, bromo-, hydroxy-, methoxy- and phenylacetaldehyde, revealed that methoxyacetaldehyde (MAA) produced strong fluorescence with Trp under milder conditions in terms of acidity, temperature and reaction time than CAA (unpublished results).

It is the purpose of this paper to describe the establishment of a practical as well as highly sensitive and selective method for the determination of Trp by a fluorescent derivation with MAA. The method was successfully applied to the determination of free and total tryptophan in serum. The elucidation of the structure of fluorescent products of the proposed method is also described.

Experimental

Materials Trp, indole-3-acetic acid, L-dopa, dopamine hydrochloride and tryptamine hydrochloride were obtained from Nakarai Tesque, Inc. (Kyoto, Japan); MAA (87%) was from Tokyo Chemical Industry, Ltd. (Tokyo, Japan); 5-hydroxy-L-tryptophan, 5-hydroxyindole-3-acetic acid, norharman hydrochloride (β -carboline), L-epinephrine and L-nor-epinephrine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); 5-hydroxytryptamine-creatinine sulfate complex was from Merck (Darmstadt, Germany). Standard human serum was from Nihon Shoji

(Osaka, Japan). All other reagents and solvents were of an analytical reagent grade. 1-Methoxymethyl- β -carboline was synthesized by the method of Bradsher.¹⁵⁾

Apparatus All fluorescence measurements were made with a Hitachi F-3010 fluorescence spectrophotometer. The excitation and emission band passes used were 5 and 10 nm, respectively. The spectra were uncorrected for wavelength.

Standard Procedure One ml of a buffer solution (0.1 M citric acid–0.2 M disodium hydrogenphosphate; pH 2.75) containing 0.3% MAA and 0.4 mM NaNO₂ was added to 50 μ l of a sample solution. The mixture was heated at 80 °C for 15 min and the fluorescence was measured at Ex. 253 nm and Em. 450 nm.

Results and Discussion

Excitation and Emission Spectra The excitation and emission spectra of Trp-adduct with MAA shown in Fig. 1 were almost identical with those obtained from the CAA method.¹³⁾ The determination of Trp by the present method was therefore carried out at the excitation and emission maxima, 253 and 450 nm, respectively.

Reaction Conditions Reaction conditions described below were established using 250 pmol/50 μ l of Trp.

It is shown in Fig. 2 that the fluorescence intensity was maintained at the highest level between pH 2.5 and 3.0, but it decreased significantly at other pHs. On the basis of this observation, pH 2.75 (0.1 M citric acid–0.2 M disodium hydrogenphosphate) was chosen as the pH of the buffer.

Fig. 1. Excitation and Emission Spectra of the Fluorescent Product

Fig. 2. Effect of pH on the Fluorescence Intensity

Fig. 3. Effect of Reaction Time and Temperature on the Fluorescence Intensity

○, 80 °C; ●, 100 °C.

As it was found that FeCl_3 and $\text{K}_3[\text{Fe}(\text{CN})_6]$ were effective in stimulating the reaction in Denckla and Dewey's method¹²⁾ where formaldehyde was used, oxidizing agents such as NaNO_2 , $\text{K}_3[\text{Fe}(\text{CN})_6]$ and $\text{K}_2\text{Cr}_2\text{O}_7$ were tested for their stimulating effect on the present method where MAA was used. Among these oxidizing agents, the addition of NaNO_2 decreased the reaction time significantly. It was also found that the fluorescence intensity of Trp reached a maximum level with 0.3 mM NaNO_2 , and the level was maintained thereafter by the standard procedure. Thus, 0.4 mM NaNO_2 was used in the standard procedure.

As the fluorescence intensity of Trp reached the maximum level with 0.3% MAA, 0.3% MAA was employed in the standard procedure.

The time course of the reaction at 80 °C or 100 °C was examined in the presence of 0.3% MAA and 0.4 mM NaNO_2 . As shown in Fig. 3, the fluorescence intensity reached the maximum level after 15 min at 80 °C and 5 min at 100 °C. In consideration of milder reaction conditions, 15 min and 80 °C were chosen as the reaction time and temperature, respectively.

Specificity of the MAA Method It was found that Trp was the only common amino acid producing fluorescence with the MAA method, while the CAA method gave fluorescence with L-cystein¹³⁾ in addition to Trp. Phenylethylamines are known to produce fluorescent isoquinoline derivatives with formaldehyde¹⁶⁾ under acidic conditions, but phenylethylamines such as L-dopa, dopamine, L-epinephrine and L-norepinephrine produced only marginal fluorescence with MAA under the conditions employed in the standard procedure. Table I summarizes the excitation

TABLE I. Excitation and Emission Maxima of Native and MAA-Induced Fluorescence and Relative Fluorescence Intensities of Indole Compounds and Phenylethylamines

	Ex. (nm)	Em. (nm)	RFI ^{b)}
Native fluorescence			
Indole-3-acetic acid	278	357	15.5
5-Hydroxyindole-3-acetic acid	279	340	0
Induced fluorescence			
L-Tryptophan ^{a)}	253	450	100
Tryptamine ^{a)}	251	450	1.9
5-Hydroxy-L-tryptophan	253	450	19
5-Hydroxy-L-tryptamine	352	450	20.8
L-Dopa	251	504	25
Dopamine	253	494	8
L-Epinephrine	254	504	0.7
L-Norepinephrine	253	501	2.5

a) 250 pmol/50 μl instead of 50 nmol/50 μl .b) $\text{RFI} = \frac{\text{FI of samples at 50 nmol/50 } \mu\text{l}}{\text{FI of Trp at 250 pmol/50 } \mu\text{l}} \times 100$

All fluorescence intensities (FI) were measured at Ex. 253 nm, Em. 450 nm.

TABLE II. Recovery of Trp in the Presence of Tryptophan Metabolites and Phenylethylamines

Added 25 nmol/50 μl	Recovery of Trp (250 pmol/50 μl), %
Tryptamine ^{a)}	112.7
Indole-3-acetic acid	88.7
5-Hydroxy-L-tryptophan	101.7
5-Hydroxy-L-tryptamine	86.7
5-Hydroxyindole-3-acetic acid	79.2
L-Dopa	96.7
Dopamine	100.2
L-Epinephrine	62.5
L-Norepinephrine	91.4

25 nmol/50 μl of tryptophan metabolites or phenylethylamines were added to 250 pmol/50 μl of tryptophan. a) 1 nmol/50 μl instead of 25 nmol/50 μl .

and emission maxima of the MAA-induced fluorescence of phenylethylamines, Trp and its metabolites. Tryptamine induced substantial fluorescence with MAA but its relative fluorescence intensity was 1.9% that of Trp at equimolecular amounts (250 pmol/50 μl) under the assay conditions. Phenylethylamines and other Trp metabolites except indole-3-acetic acid and 5-hydroxyindole-3-acetic acid produced only weak fluorescence with MAA, even at higher concentrations (50 nmol/50 μl). Native fluorescence of indole-3-acetic acid was observed under standard assay conditions.

Addition Recovery Recovery tests for Trp were performed by adding various concentrations of phenylethylamines and Trp metabolites to 250 pmol/50 μl of Trp. As shown in Table II, each 25 nmol/50 μl of 5-hydroxy-L-tryptamine, indole-3-acetic acid, 5-hydroxy-3-acetic acid, L-epinephrine and L-norepinephrine reduced the recovery of Trp significantly. However, the effect of these compounds may not be a serious problem in the determination of serum Trp with the MAA method because they are present in minute quantities in the blood.¹⁷⁾ The levels of serum 5-hydroxy-L-tryptamine and L-epinephrine are reported to be 74 and 2.6 nM, respectively, and that of plasma L-norepinephrine is 5.7 nM.¹⁸⁾ It was confirmed that no effect on the recovery of Trp was observed at these concentrations.

Fig. 4. Selected Ion Chromatograms

Working Curve A linear working curve was obtained by a standard procedure from 10 pmol/50 μ l to 500 pmol/50 μ l of Trp. The limit of determination for Trp was 10 pmol/50 μ l. The reproducibility of the MAA method was determined by assaying 250 pmol/50 μ l of Trp, and the relative standard deviation of the measurement ($n=5$) was 1.5%.

Total Trp The application of the MAA method for clinical analyses was attempted by the use of a pooled human serum. Total Trp could be determined by measuring the concentration of Trp in the supernatant after deproteinization with trichloroacetic acid (TCA) because TCA dissociates the Trp-protein complex.¹⁹⁾ A linear working curve for total Trp was obtained using a pooled human serum to which was added 10 to 100 μ M Trp. One hundred microliters of a pooled human serum, to which was added 50 μ M Trp, was deproteinized with 500 μ l of 6% TCA, and the recovery of Trp was obtained by the MAA method. The recovery of total Trp was 98.8%.

Free Trp As Trp is bound to albumin in human serum, the determination of free Trp requires its separation from a fraction of bound Trp.²⁰⁾ Ultrafiltration with ADVANTEC ultrafilter UP-20 was employed for this purpose. Addition recovery of free Trp was examined by adding 10 μ M of Trp to the ultrafiltrate of a pooled human serum. A linear working curve for free Trp was obtained using the ultrafiltrate of a pooled human serum to which was added 1 to 15 μ M of Trp. The recovery of free Trp was 99.1% from the ultrafiltrate, which was diluted twofold with distilled water prior to analysis by the MAA method.

Fluorescent Compound Fluorescent products of the reaction between Trp and MAA under the conditions employed by the proposed method were examined by a combination of a precolumn derivatization method and a liquid chromatography-mass spectrometry (Hewlett Packard HP 1090 LC-MS). A reaction mixture of Trp and MAA was evaporated to dryness and the residue was extracted with acetonitrile to remove salts. The acetonitrile phase was injected onto a column of LC-MS. Fragments with m/z 169 and 213 which correspond to two peaks of fluorescent products were observed at retention times of 10.4 and 15.4 min, respectively, under the conditions for the thermospray ionization mode of LC-MS, and the selected ion chromatograms obtained by LC-MS are shown in Fig. 4. Fragments at m/z 169 and 213 were assigned as the MH^+ of β -carboline and that of 1-methoxymethyl- β -carboline, respectively. The more positive identification of the fluorescent products was obtained by co-chromatography using authentic β -carboline and synthesized 1-methoxy-

Fig. 5. Chromatograms of (A) Fluorescent Products of the Standard Method and (B) Authentic β -Carboline and 1-Methoxymethyl- β -carboline

Chromatographic conditions: column, TSK gel ODS 80 TM (4.6 mm i.d. $\times$ 15 cm); column temperature, 40 $^{\circ}$ C; eluent, 0.05 M $CF_3COOH + NH_4OH$ (pH 2.5); $CH_3CN=8:2$ (0.7 ml/min); detection, fluorescence (Ex. 255 nm, Em. 450 nm). Chromatograms of (B): 10.9 min, β -carboline; 16.0 min, 1-methoxymethyl- β -carboline.

methyl- β -carboline (Fig. 5). Thus it was confirmed that the fluorescent products by the proposed method were β -carboline and 1-methoxymethyl- β -carboline.

The main fluorescence product of the MAA method was expected to be 1-methoxymethyl- β -carboline, but it was revealed that three-fifths of the total fluorescence intensities was due to β -carboline in the present experiment. An almost identical result was obtained by HPLC using $K_3[Fe(CN)_6]$ as an oxidizing agent in place of $NaNO_2$. Therefore, it was confirmed that $NaNO_2$ acted only as an oxidizing agent without the nitrosation of tryptophan²¹⁾ during the process of reaction by the MAA method. Presumably, β -carboline was produced by demethoxymethylation of the intermediate products because the HPLC analysis performed under the conditions described in Fig. 5 was unable to show that β -carboline was produced by heating an authentic 1-methoxymethyl- β -carboline in the reagent solution of the MAA method.

In conclusion, the proposed MAA method was found to be highly sensitive and selective for the determination of Trp as compared to Denckla and Dewey's method or its modification²²⁾ in which formaldehyde was used for the determination of Trp. Our results in terms of sensitivity and selectivity were almost comparable with those obtained by a phenylglyoxal method¹⁴⁾ or CAA method, both of which were also bases on the Pictet-Spengler reaction. The reaction conditions of the MAA method, however, are milder, as it requires heating for 15 min at 80 $^{\circ}$ C at pH 2.75 while methods using formaldehyde, phenylglyoxal and CAA require heating for 30 to 60 min at 100 $^{\circ}$ C in 0.05 M HCl or stronger acids.

Application of the MAA method to a single-pump, post-column derivation method for HPLC analysis of Trp in human serum is under way and will be published elsewhere.

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References and Notes

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