High-performance Liquid Chromatographic Measurement of Tryptophan in Blood, Tissues, Urine, and Foodstuffs with Electrochemical and Fluorometric Detections

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A rapid and convenient measurement of tryptophan in whole blood, serum, liver, brain, urine, and alkaline hydrolysates of proteins and foodstuff was done by high-performance liquid chromatography. The sample preparation was simply homogenized or mixed in a 5% trichloroacetic acid solution and a sample of the supernatant was injected onto a column after filtration with a 0.45-μm filter. The method used a Chemcosorb 5-ODS-H column (particle size, 5 μm, 150 × 4.6 mm i.d.) eluted with 20 mM potassium dihydrogen phosphate (pH adjusted to 3.7 by the addition of phosphoric acid) containing 1 g/l of sodium heptane sulfonate and 3 mg/l EDTA·2Na–acetonitrile (93:7, v/v) at a flow rate of 1.5 ml/min. The tryptophan contents in whole blood, serum, liver, and brain were electrochemically estimated at +1000 mV *vs.* Ag/AgCl, the detection limit being 0.2 pmol (40.84 pg) at a signal-to-noise ratio of 5:1. The tryptophan contents in urine, proteins, and foodstuff were fluorometrically estimated with an excitation wavelength of 280 nm and with an emission wavelength of 340 nm, the detection limit being 20 pmol (4.08 ng) at a signal-to-noise ratio of 5:1. Tryptophan was eluted at about 10.5 min. The total analysis time was about 12 min.

Tryptophan is an essential amino acid and also the precursor of the neurotransmitter serotonin, behavior-active trace amines like tryptamine, and a vitamin, nicotinic acid and nicotinamide. Numerous methods have been developed for the separation and measurement of tryptophan and its metabolites. Among these methods, high-performance liquid chromatography (HPLC) has become one of the most convenient methods for the measurement of tryptophan and its metabolites. However, for the measurement of only tryptophan, some of these methods are complicated and time-consuming[1-5] and some of these methods are applicable to only one of these biological materials such as brain,[3,6,7] plasma and serum,[5,8-12] urine,[1,4,13] or foodstuff.[14,15] That is, no HPLC method for the measurement of tryptophan in biological materials such as blood, urine, tissues and foodstuff appeared to be entirely adequate. We report an isocratic reversed-phase ion-pair HPLC method using electrochemical and fluorometric detections that permits the measurement of tryptophan in biological materials and foodstuff in less than 12 min. The sample preparation was simply homogenized or mixed in a 5% trichloroacetic acid solution and a sample of the supernatant was injected onto the column after filtration with a 0.45-μm filter.

Materials and Methods

Chemicals. Tryptophan was obtained from Wako Pure Chemical Co., (Osaka, Japan). Sodium heptane sulfonate was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Lysozyme (chicken egg white, Grade I) and albumin (chicken, Fraction V) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Wheat flour (hard wheat bread flours) was obtained from Nisshin Flour Milling Co (Tokyo, Japan). All the other chemicals used were of the highest commercial grade available. The concentration of tryptophan in water was calculated using the value for the molar absorptivity of 4930 $M^{-1} \cdot cm^{-1}$.

Analysis. The apparatus consisted of an LC 4-A liquid chromatograph with an Irica E-502 electrochemical detector with a glassy carbon electrode (Irica, Kyoto, Japan) and a RF-540 spectrofluorophotometer equipped with an LC-flow-cell unit (Shimadzu, Kyoto, Japan), a Model 7125 syringe loading sample injector (Rheodyne, Cotati, CA, U.S.A.), a Chemcosorb 5-ODS-H column (150 × 4.6 mm i.d., particle size 5 μm; Chemco Scientific Co., Osaka, Japan). The mobile phase was a mixture of a degassed solution of 20 mM potassium dihydrogen phosphate (pH adjusted to 3.7 by the addition of phosphoric acid) containing 1 g/l of sodium heptane sulfonate and 3 mg/l of EDTA·2Na–acetonitrile (93 : 7, v/v) and was used at a flow rate of 1.5 ml/min. The column temperature was maintained at 25°C. Tryptophan contents in whole blood, serum, brain, and liver were electrochemically measured at +1000 mV *vs.* Ag/AgCl and tryptophan contents in urine, alkaline hydrolysates of protein, and foodstuff were fluorometrically measured at an excitation wavelength of 280 nm (10-nm bandpass) and at an emission

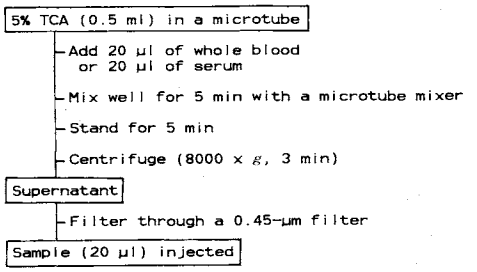

Fig. 1. Procedure for Extracting Tryptophan from Whole Blood and Serum.
TCA = trichloroacetic acid.

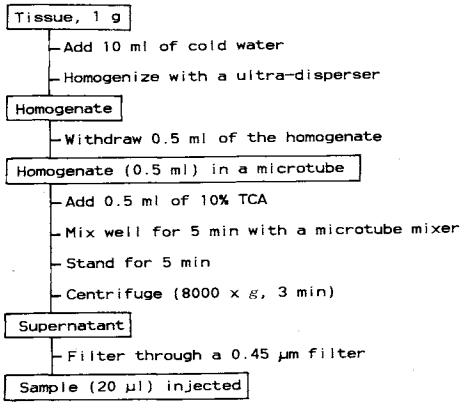

Fig. 2. Procedure for Extracting Tryptophan from Tissues.

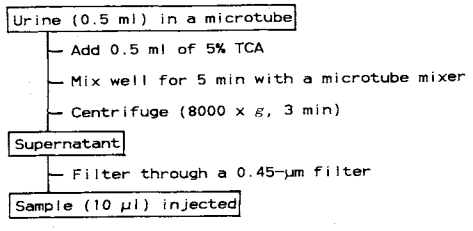

Fig. 3. Procedure for Extracting Tryptophan from Urine.

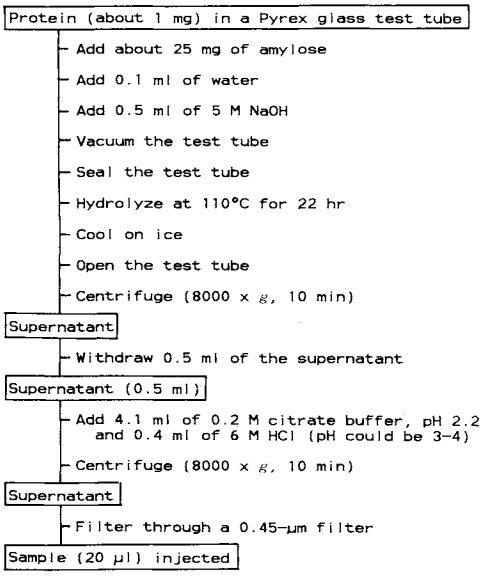

Fig. 4. Procedure for Alkaline Hydrolysis of Protein and Extracting Tryptophan from the Alkaline Hydrolysate.

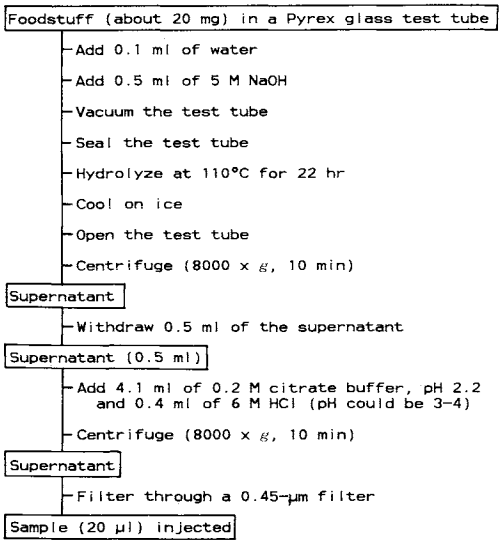

Fig. 5. Procedure for Alkaline Hydrolysisof Foodstuff and Extracting Tryptophan from the Alkaline Hydrolysate.

wavelength of 340 nm (10-nm bandpass). The HPLC system was interfaced with a Shimadzu Chromatopac C-R3A for data processing.

Extraction of tryptophan from biological materials and foodstuff. The procedure for extracting tryptophan from whole blood and serum in humans and rats, tissues such as liver and brain in rats, urine in humans and rats, and alkaline hydrolysate of protein such as lysozyme and albumin, and alkaline hydrolysate of foodstuff such as wheat flour are shown in Figs. 1, 2, 3, 4, and 5, respectively. The procedure for hydrolysis of protein in NaOH solution was based on the methods of Hugli and Moore[16] and Sato *et al.*[17]

Results and Discussion

Detection method

Tryptophan contents in whole blood, serum, liver, and brain were measured by electrochemical detection because the sensitivity to tryptophan was 100 times higher than by fluorometric detection, the tryptophan in each extract being eluted without interfering peaks. However, tryptophan contents in urine and an extract of alkaline hydrolysate of protein could not be detected by electrochemical detection because this tryptophan was eluted with interfering peaks. Accordingly, tryptophan contents of urine and protein were measured by the fluorometric method. The tryptophan contents in an extract of the alkaline hydrolysate of wheat flour could be measured by both detections such as electrochemical and fluorometric detections.

Calibration curve and limit of detection

The calibration curve for tryptophan was linear in the range of 0.2 pmol–1000 pmol (electrochemical detection) and 20 pmol–20-nmol (fluorometric detection) per injection. The coefficient of variation (C.V.) of the peak area of tryptophan (2 pmol per injection; five injection) was 1.6% when tryptophan was electrochemically measured and that (200 pmol per injection; five injection) was 1.3% when tryptophan was fluorometrically measured. The low limit of detection were 0.2 pmol (40.84 pg) with the electrochemical detection and 20 pmol (4.08 ng) in fluorometrical method at a signal-to-noise ratio of 5 : 1.

Precision and recovery of tryptophan from biological materials

To measure the precision of the method, the measurement for tryptophan contents of samples such as whole blood, serum, liver homogenate, brain homogenate, and urine in rats (see, Table IV) were repeated five times. As shown in Table I, all the C.V. were around 2%, indicating that the reproducibility of this

Table I. Precision and Recoveries of Added Tryptophan (Trp) from Whole Blood, Serum, Liver, Brain, and Urine in Rats

Added Trp (nmol)	Found	C.V. (%)	Recovery (%)
	(nmol/20 μl of whole blood)		
0	1.387±0.029	2.1	—
5*1	6.168±0.142	2.3	95.6*2
	(nmol/20 μl of serum)		
0	2.497±0.055	2.2	—
5*1	7.513±0.302	4.0	100.3*3
	(nmol/500 μl of liver homogenate)*4		
0	3.292±0.112	3.4	—
10*5	13.159±0.276	2.1	98.7*6
	(nmol/500 μl of brain homogenate)*4		
0	1.443±0.038	2.6	—
10*5	11.007±0.273	2.5	95.6*7
	(nmol/500 μl of urine)*8		
0	22.32±0.33	1.5	—
100*9	122.49±2.18	1.8	100.2*10

Values are means±SD for five times.

*1 A 5-μl sample of 1 mM tryptophan was added to 20 μl of whole blood or serum.

*2 $(6.168-1.387)/5\times100=95.6\%$.

*3 $(7.513-2.497)/5\times100=100.3\%$.

*4 Liver or brain homogenate was prepared as in Fig. 2.

*5 A 10-μl of sample of 1 mM tryptophan was added to 500 μl of liver or brain homogenate.

*6 $(13.159-3.292)/10\times100=98.7\%$.

*7 $(11.007-1.443)/10\times100=95.6\%$.

*8 Urine was diluted with 0.1 M HCl to make up to 25 ml per day.

*9 A 10-μl of sample of 10 mM tryptophan was added to 500 μl of urine.

*10 $(122.49-22.32)/100\times100=100.2\%$.

method was very high. The test samples were prepared by adding 5 nmol, 10 nmol, and 100 nmol of tryptophan to 20 μl of whole blood or serum, to 500 μl of the organ tissue homogenate, and to 500 μl of urine respectively, according to procedures shown in Figs. 1, 2, and 3. High recoveries of tryptophan from these samples were obtained (Table I).

Table II. RECOVERY OF TRYPTOPHAN PRESENT IN SAMPLES OF PURIFIED PROTEINS

	Theoretical value (mol/mol of protein)	Found (mol/mol of protein)	Found theoretical (%)
Lysozyme*1	6	5.95±0.17	99.2±2.9
Albumin*2	3	2.96±0.04	98.5±1.6

Values are means±SD for triplicate analyses of two hydrolysates.

*1 The concentration of lysozyme (chicken egg white) in water was calculated using $OD^{1\%}_{282\,nm}=26.4$.

*2 The concentration of albumin (chicken) in 0.05 M NaOH was calculated using $OD^{1\%}_{280\,nm}=7.5$.

Recovery of tryptophan present in protein

The recovery of tryptophan contents in

Table III. STABILITY OF TRYPTOPHAN IN A TRICHLOROACETIC ACID EXTRACTS OF BIOLOGICAL MATERIALS IN RATS

	0 hr	24 hr	48 hr	72 hr
Whole blood	100	102.7	101.5	102.7
Serum	100	98.7	100.1	100.1
Liver	100	100.6	104.9	103.3
Brain	100	100.9	99.1	104.0
Urine	100	101.9	100.7	103.5

Values are an average of two samples.
The extracts of trichloroacetic acid were kept at 0–4°C.

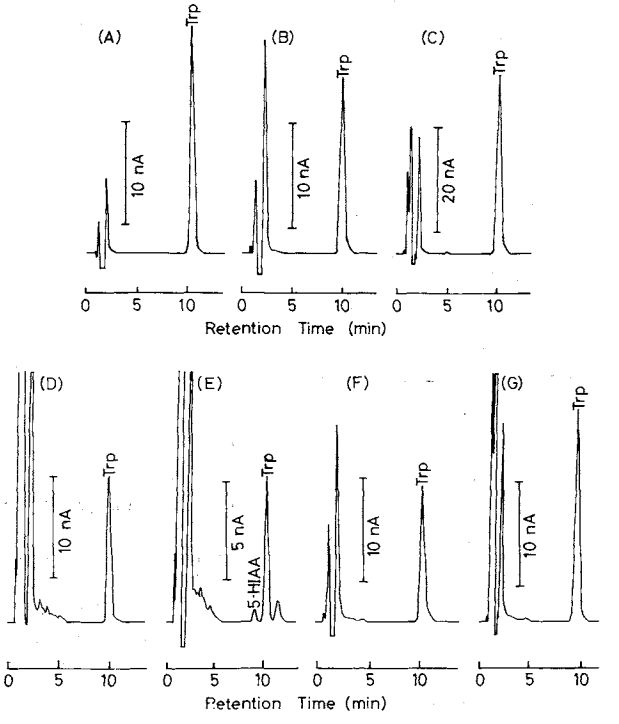

Fig. 6. Chromatograms (Electrochemical Detection) of (A) Standard Tryptophan in 5% Trichloroacetic Acid (100 pmol/10 μl injected), (B) an Extract of Rat Whole Blood (sample size was 20 μl containing 76.0 pmol of tryptophan), (C) an Extract of Rat Serum (sample size was 20 μl containing 158.7 pmol of tryptophan), (D) an Extract of Rat Liver (sample size was 20 μl containing 63.5 pmol of tryptophan), (E) an Extract of Rat Brain (sample size was 20 μl containing 31.5 pmol of tryptophan); 5-HIAA = 5-hydroxyindole-3-acetic acid, (F) an Extract of Human Whole Blood (sample size was 20 μl containing 57.1 pmol of tryptophan), and (G) an Extract of Human Serum (sample size was 20 μl containing 92.7 pmol of tryptophan).

samples of purified proteins were observed to be 99.2±2.9% for lysozyme (triplicate analysis of two hydrolysates) and 98.5±1.6% for albumin (triplicate analysis of two hydrolysates) as shown in Table II. These results indicated that the procedure for the alkaline hydrolysis used (Fig. 4) is suitable for measuring tryptophan in protein as reported by Hugli and Moore[16] and Sato *et al.*[17]

Stability of tryptophan in a trichloroacetic acid extract of biological materials

Since tryptophan is known to be labile in an acid solution, the stability of tryptophan in the trichloroacetic acid extracts of whole blood, serum, liver, brain, and urine was examined. As shown in Table III, the tryptophan in the acid extracts from whole blood, serum, liver, brain, and urine was stable within 72 hr when the samples were stored at 0–4°C. Geeraerts *et al.*[18] reported that tryptophan and its metabolites such as kynurenine, serotonin, 5-hydroxytryptophan, 5-hydroxyindole-3-acetic acid, and kynurenine acid in urine were stable within 7 days when the urine was stored at 4°C and the pH was adjusted to 2. Sato *et al.*[17] reported that the tryptophan in a hydrolysate of lysozyme was stable at least for 14 days when the hydrolysate was stored at 5°C and the pH was adjusted to 4.2. In the present experiment, the tryptophans in alkaline hydrolysates of lysozyme, albumin, and wheat flour were stable at least for 7 days when the alkaline hydrolysates was prepared according to the procedure as shown in Figs. 4 and 5, and stored at 0–4°C.

Application of the method

Electrochemical detection. The chromatogram of the standard tryptophan is shown in Fig. 6-A, tryptophan being eluted at about 10.5 min. The chromatograms of the extracts of rat whole blood, rat serum, rat liver, rat brain, human whole blood, and human serum are shown in Fig. 6-B, -C, -D, -E, -F, and -G, respectively. Tryptophans in these samples were characterized on the basis of these retention time, the total HPLC analysis being about 12 min. When sodium heptane sulfonate was omitted from the mobile phase, tryptophan was eluted at about 3.5 min with interfering peaks.

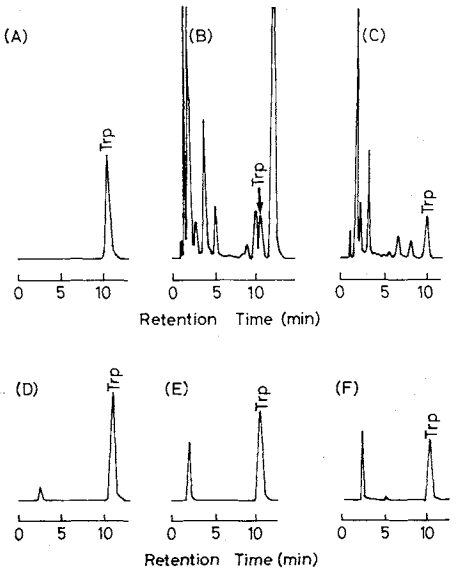

Fig. 7. Chromatograms (Fluorometric Detection) of (A) Standard Tryptophan in 5% Trichloroacetic Acid (500 pmol/10 μl injected; Shimadzu Chromatopac C-R3A, attenuation 4), (B) an Extract of Rat Urine (sample size was 10 μl containing 171 pmol of tryptophan; attenuation 4), (C) an Extract of Human Urine (sample size was 10 μl containing 463 pmol of tryptophan; attenuation 5), (D) an Extract of Alkaline Hydrolysate of Lysozyme (sample size was 20 μl containing 1186 pmol of tryptophan; attenuation 5), (E) an Extract of Alkaline Hydrolysate of Albumin (sample size was 20 μl containing 506 pmol of tryptophan; attenuation 4), and (F) an Extract of Alkaline Hydrolysate of Wheat Flour (sample size was 20 μl containing 341 pmol of tryptophan; attenuation 4).

Fluorometric detection. The chromatogram of the standard tryptophan is shown in Fig. 7-A. The chromatograms of the extract of rat urine, human urine, alkaline hydrolysate of lysozyme, alkaline hydrolysate of albumin, and alkaline hydrolysate of wheat flour samples are shown in Fig. 7-B, -C, -D, -E, and -F, respectively. Tryptophans in these samples were characterized on the basis of these retention time and the entire excitation and emission spectra between 250 nm and 400 nm.

Tryptophan contents of whole blood, serum, liver, brain, and urine in rats fed with a 20%

Table IV. TRYPTOPHAN CONTENTS IN BIOLOGICAL MATERIALS AND FOODSTUFF

	Tryptophan content	Values from the literature
Rat[1]		
Whole blood (nmol/ml)	116.5±13.6	
Serum (nmol/ml)	185.7±11.6	110.3±10.3[19)a]
Liver (nmol/g)	62.4±3.1	64.2±10.8[19)]
Brain (nmol/g)	33.8±3.8	23.7±2.7,[3)] 33.4±3.2[20)]
Urine (nmol/day)	644±83	*ca.* 600[21)]
Human (women)[2]		
Whole blood (nmol/day)	62.3±4.7	
Serum (nmol/ml)	90.3±4.2	46.3±1.6,[8)] 62.5±13.5,[2)] 55.8±10.2,[22)] 58.0±15.4,[23)] 56.7–77.1[24)]
Urine (μmol/day)	50.2±27.2	80.4,[19)] 37.4±10.8,[28)]
Foodstuff[3]		
Wheat flour (mg/100 g dry matter)	158±5.75	183±41,[25)] 149,[26)] 173,[15)] 139,[15)] 144[15)]

[a] Reference number.

[1] Values are means±SD for five rats. Male rats of the Wistar strain (8 week old; body weight, about 240 g) were obtained from Clea Japan Inc. (Tokyo, Japan) and placed immediately in individual metabolic cages (CT-10; Clea Japan). The rats were fed with a 20% casein diet [casein, 20%; α-cornstarch, 46%; sucrose, 23%; corn oil, 5%; mineral mixture (Oriental's ration),[27)] 5%; vitamin mixture (Oriental's ration),[27)] 1%] *ad libitum* for 10 days. Urine samples (48-hr urine) were collected in bottles containing 1 ml of 1 M HCl from 10:00 hr on the 9th day to 10:00 hr on the 11th day and stored at −25°C until analyzed. The rats (body weight, about 280 g) were killed by decapitation at around 10:00 hr on the 11th day, the blood was collected in tubes without anticoagulant, a part of whole blood was prepared as shown in Fig. 1 for measureing tryptophan. The livers and brains were removed and prepared as shown in Fig. 2. The clotted blood samples were centrifuged at 2000×*g* for 10 min, and serum was collected and prepared as shown in Fig. 1.

[2] Values are means±SD for six women (21–25 years old). Human blood was collected before lunch from the antecubital vein in tubes without anticoagulant, a part of whole blood was prepared as shown in Fig. 1, and the remaining blood was allowed to clot for 30 min at room temperature. The clotted blood samples were centrifuged at 2000×*g* for 10 min and serum was collected and prepared as shown in Fig. 1. Urine was collected from the second excretion on the day to the first excretion on the next day and stored at −25°C until analyzed.

[3] Values are means±SD for triplicated analyses of two alkaline hydrolysates.

casein diet, and whole blood, serum, and urine in human, and wheat flour are given in Table IV.

Under the HPLC conditions used in this study, the tryptophan metabolites such as xanthurenic acid (about 1.5 min), 3-hydroxykynurenine (2.1 min), kynurenic acid (2.5 min; detected with an excitation wavelength of 344 nm and an emission wavelength of 398 nm: did not react electrochemically and no detectable peak at an excitation wavelength of 280 nm and an emission wavelength of 340 nm), 5-hydroxytryptophan (3.2 min), kynurenine (4.5 min), 3-hydroxyanthranilic acid (5.2 min), 5-hydroxyindole-3-acetic acid (9.5 min), anthranilic acid (16 min), serotonin (34 min), and indole-3-acetic acid (60 min) were eluted, but tryptamine was not eluted. So, these tryptophan metabolites did not disturb the detection of tryptophan, and these metabolites except for 5-hydroxyindole-3-acetic acid (1972±286 pmol/g brain; mean±SD for five rats) in brain were not below the limits of detection under these preparation methods.

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