FLUOROMETRIC DETERMINATION OF 5-HYDROXYINDOLE DERIVATIVES
BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH COBALT(II)
CHLORIDE, SODIUM CARBONATE, AND SODIUM HYDROXIDE

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High-performance liquid chromatographic determination of 5-hydroxyindole derivatives was established. The postcolumn derivatization with sodium carbonate-sodium hydroxide-cobalt(II) chloride reagent allowed the determination of pmol level of the substances. This technique was applied to the determination of 5-hydroxyindoleacetic acid in human urine.

The importance of the neuroregulatory and hormone action of serotonin has become well established¹⁾. Although the biochemical pathway for central and peripheral metabolism of serotonin has been elucidated, the dynamics with respect to normal and stressed conditions are being investigated. Recently, high performance liquid chromatography (HPLC) methodologies have provided a rapid and simultaneous determination of several indole compounds. The combination of HPLC with flow through fluorometric detection of 5-hydroxyindoles²⁾³⁾ seems to give significant improvement of sensitivity. Native fluorescence of 5-hydroxyindoles and fluorescence reaction with o-phthalaldehyde have been used for this purpose. However, these fluorescences are not specific to 5-hydroxyindoles, and we are prompted to develop more specific reaction.

Recently, we have developed a method for the fluorometric determination of tryptophan and its metabolite in serum based on the fact that these compounds gave fluorescence on reaction with hydrogen peroxide in alkaline solution⁴⁾. But 5-hydroxyindoles such as serotonin and 5-hydroxyindoleacetic acid (5-HIAA) give no fluorescence on the reaction with hydrogen peroxide. In the present study, 5-hydroxyindoles were found to give fluoresce on heating under alkaline conditions. This reaction was applied to the HPLC determination of 5-hydroxyindoles in human urines.

EXPERIMENTAL

5-Hydroxyindoles and LiChrosorb RP-8 (5 μ m) were purchased from Sigma Chemical (St. Louis, MO, U.S.A.), and E. Merck (Darmstadt, G.F.R.), respectively. Other chemicals used were obtained from Wako Pure Chemical (Osaka, Japan).

Pretreatment of human urine⁵⁾

Human urine was collected for 24 h. To 1/100 volume of the collected urine was added 0.85 ml of perchloric acid and the mixture was made up to 25 ml with distilled water. An aliquot (2 ml) of the diluted sample solution was applied on a column of Sephadex G-10 (3 cm x 1 cm I.D.). The column was washed with 20 ml of 0.01 M hydrochloric acid and then 5-HIAA was eluted with 10 ml of 0.05 M phosphate buffer (pH 7.7). The effluent was submitted to both the manual and chromatographic determination described below.

Manual procedure for the determination of 5-hydroxyindoles

To 0.5 ml of sample solution was added 2.5 ml of aqueous 0.15 mM sodium carbonate-0.12 M sodium hydroxide-12 μ M cobalt(II) chloride solution. The reagent solution was prepared just before used. The test tube was incubated in a water bath for 30 min at 80°C. After cooling the tube in running water, the fluorescence intensity of the mixture was measured at 460 nm with excitation at 360 nm with a Hitachi Fluorescence Spectrophotometer Model 650-10S.

HPLC of 5-hydroxyindoles

Figure 1 shows a schematic diagram of this system. The separation was

Fig. 1 Flow diagram of HPLC system for 5-hydroxyindoles using sodium carbonate-sodium hydroxide-cobalt(II) chloride as postcolumn reagent

P-1: high pressure pump (flow rate, 0.75 ml/min); PG: pressure gauge; VS: valve universal injector; C: LiChrosorb RP-8 column (50 mm x 4 mm I.D., particle size 5 μ m); RC: reaction coil (15 m x 0.5 mm I.D., stainless steel) maintained at 180°C in a silicon oil bath; CC: cooling coil (70 cm x 0.5 mm I.D., stainless steel); FD: fluorescence detector; P-2: reagent pump (flow rate, 0.5 ml/min); R: recorder.

achieved on a reversed phase column (50 mm x 4 mm I.D.), which was packed with LiChrosorb RP-8 (particle size 5 μ m) by slurry packing method⁶⁾ using SNK High Performance Column Packer Model SPC-102 (Sanuki Kogyo Tokyo, Japan). The mobile phase is 50 mM acetate buffer (pH 4.4) containing 0.1 M sodium sulfate and 0.5 μ M cobalt(II) chloride. A pressure pump with a valve universal injector (Sanuki Kogyo, Tokyo, Japan) is used to deliver mobile phase at a flow rate of 0.75 ml/min. The eluate was mixed with 0.5 M sodium carbonate-2 M sodium hydroxide in the reaction coil at 180 $\pm$ 3°C to form fluorescent derivatives of 5-hydroxyindoles. The temperature of the silicon oil bath was maintained at 180 $\pm$ 3°C with a mantle

heater. The fluorescence was monitored at 460 nm with excitation at 360 nm with a Hitachi Model 650-10S fluorescence spectrophotometer with a 40 μ l flow cell.

RESULTS AND DISCUSSION

Figure 2 shows excitation and emission spectra of serotonin reacted with the sodium carbonate-sodium hydroxide-cobalt(II) chloride reagent. Maxima of excitation were at 260, 315, and 360 nm, and maximum of emission was at 460 nm. The fluorescence intensity at 460 nm was enhanced in the presence of sodium hydroxide. Assuming that the relative fluorescence intensity was 100 when the reagent containing 0.1 M sodium carbonate, 0.1 M sodium hydroxide and 10 μ M cobalt(II) chloride was used, it decreased to 56 in the absence of sodium

Fig. 2 Fluorescence excitation and emission spectra of serotonin allowed to react with sodium carbonate-sodium hydroxide-cobalt(II) chloride reagent

Table 1 Effect of addition of 10 μ M metal ion on the fluorescence intensity of serotonin (2.35 nmol)

Compound	Relative fluorescence intensity*
None	48.5
Cobalt(II) chloride	100
Copper(II) sulfate	47.6
Magnesium(II) chloride	22.6
Manganese(II) acetate	46.5
Aluminium(III) chloride	42.2
Zinc(II) chloride	28.3

* Measured at 460 nm with excitation at 360 nm.

Intensity for cobalt(II) chloride was taken as 100.

hydroxide. Table 1 shows the effect of the addition of metal ion on the fluorescence intensity of serotonin. Addition of cobalt(II) chloride markedly increased the fluorescence intensity, whereas other metal ions did not. Figure 3 shows the fluorescence intensity of serotonin relative to the concentration of cobalt(II) chloride. A plateau was observed between 10 and 100 μM of cobalt(II) chloride.

Figure 4 shows the fluorescence intensity of serotonin as a function of reaction time at various reaction temperatures. The fluorescence intensity reached to a plateau after 20 min when reacted at 80°C. Accordingly, the reaction time of 30 min was adopted in the manual procedure.

Fig. 3 Effect of concentration of cobalt (II) chloride on the fluorescence intensity of serotonin

Fig. 4 Effects of reaction time and temperature on the fluorescence intensity of serotonin

●: 20°C; ▲: 50°C; □: 70°C; ○: 80°C; ■: in a boiling water bath.

Measured at 460 nm with excitation at 360 nm.

The calibration curves for N-acetylserotonin ($y = -0.43 + 98.5x$, $r = 1.000$), 5-HIAA ($y = -1.75 + 85.1x$, $r = 0.998$), and 5-hydroxytryptophol ($y = 3.14 + 62.9x$, $r = 0.999$) were linear in the range of final concentration of 30 pmol to 5.5 nmol. Those for serotonin ($y = -0.64 + 61.9x$, $r = 1.000$), 5-hydroxyindoleacetamide ($y = -0.12 + 9.0x$, $r = 1.000$), and 5-hydroxytryptophan ($y = -0.48 + 7.3x$, $r = 0.996$) were linear in the range of 120 pmol to 5.5 nmol, 260 pmol to 6.3 nmol, 225 pmol to 5.5 nmol, respectively. The relative standard deviations of these compounds (0.8 nmol, $n = 8$) were less than 3%.

Relative fluorescence intensities of serotonin in the presence of various compounds are listed in Table 2. Ascorbic acid and sodium sulfite did not interfere with the reaction of serotonin when the same amount was added to the sample. However, further addition of these compounds caused some interference. Sucrose, ammonium sulfate, and amino acids affected the reaction slightly. Glucose and fructose were found to affect the assay. Sodium sulfate, creatine,

creatinine, mannitol, and sorbitol did not interfere with the reaction.

Table 2 Fluorescence intensity of serotonin (2 nmol) developed by sodium carbonate-sodium hydroxide-cobalt(II) chloride reagent in the presence of several compounds

Compound	Amount of compound added /nmol	Relative fluorescence intensity
None	-	100*
Glycine	200	105
Alanine	200	94
Leucine	200	117
Phenylalanine	200	112
Histidine	10	112
Aspartic acid	200	108
Creatine	200	99
Creatinine	200	102
Glucose	200	19
	2	78
Fructose	200	22
	2	72
Mannitol	10	99
Sorbitol	10	104
Sucrose	200	107
Ascorbic acid	200	57
	2	98
Sodium sulfite	200	68
	20	98
Sodium sulfate	10	104
Ammonium sulfate	10	110

*Intensity on this condition was taken as 100.

The above mentioned reaction was applied to the postcolumn derivatization of 5-hydroxyindoles in HPLC (Fig. 1). Elution with 50 mM acetate buffer containing sodium sulfate (pH 5.3) facilitated the separation of 5-hydroxytryptophan (capacity ratio k' : 0.94), 5-HIAA(k' : 3.69), 5-hydroxytryptophol(k' : 6.66), and N-acetylserotonin(k' : 18.33), but serotonin overlapped with 5-HIAA. When the pH of the mobile phase was lowered to 4.4, 5-hydroxytryptophan(k' : 0.94), serotonin(k' : 2.91), 5-HIAA(k' : 4.42), and 5-hydroxytryptophol(k' : 6.64) could be separated, but N-acetylserotonin was too strongly retained to elute from column. In the study described below, the mobile phase of pH 4.4 was used, because the analysis of 5-HIAA was main purpose of the present investigation. Postcolumn derivatization was carried out by mixing the alkaline solution to the eluate from separa-

Table 3 Effect of reaction condition of postcolumn derivatization for HPLC on the fluorescence intensity of 5-HIAA (50 pmol)

Reaction condition	Relative fluorescence intensity*
0.1 M Na ₂ CO ₃ + 0.8 M NaOH + 15 μM CoCl ₂	100**
0.1 M Na ₂ CO ₃ + 0.8 M NaOH	83
0.8 M NaOH + 15 μM CoCl ₂	70
0.8 M NaOH	22
0.1 M Na ₂ CO ₃ + 0.8 M NaOH + 30 μM CoCl ₂	101

* Conditions of postcolumn derivatization were described in the Experimental.

** Intensity on this condition is taken as 100.

Fig. 5 Chromatogram of 5-hydroxyindoles obtained by HPLC with postcolumn derivatization using sodium carbonate-sodium hydroxide-cobalt(II) chloride
Column: LiChrosorb RP-8 (50 mm × 4 mm I.D., particle size 5 μm); Mobile phase: 50 mM acetate buffer (pH 4.4) containing 0.1 M sodium sulfate and 0.5 μM cobalt (II) chloride (flow rate, 0.5 ml/min). Fluorescence intensity was monitored at 460 nm with excitation at 360 nm. Peaks: 1; 45 pmol of 5-hydroxytryptophan, 2; 52 pmol of 5-HIAA, 3; 56 pmol of 5-hydroxytryptophol.

Fig. 6 Typical Chromatogram of 5-hydroxyindoles in urine
Urinary sample was pretreated with Sephadex G-10. HPLC conditions are the same as in Fig. 4. Peaks: 1;5-HIAA, 2 and 3;unknown.

tion column at 180°C. Table 3 shows the relative fluorescence intensity under various reaction conditions. Reaction temperature of 180°C gave high sensitivity. Figure 5 shows a chromatogram for the mixture of 5-hydroxyindoles. Limits of detection of 5-hydroxytryptophan, 5-HIAA, and 5-hydroxytryptophol were 12 pmol, 5 pmol, 6 pmol, respectively. Standard 5-HIAA was detected as a single peak, with retention time of about 8 min. The calibration curves for 5-hydroxytryptophan ($y = -2.48 + 1.52x$, $r = 0.989$), 5-HIAA ($y = -10.6 + 5.78x$, $r = 0.999$), and 5-hydroxytryptophol ($y = -9.18 + 4.96x$, $r = 0.993$) were linear in the range of 45 pmol to 900 pmol, 15 pmol to 100 pmol, 15 pmol to 100 pmol, respectively. The relative standard deviation for 105 pmol of 5-HIAA was 2.6% ($n = 3$). Figure 6 illustrates typical chromatogram of urine sample. The urinary sample gave the peak of 5-HIAA together with those of and two unknown substances. These unknown substances were clearly separated from 5-HIAA and its derivatives.

Table 4 5-HIAA content in a urine sample and the recovery of 5-HIAA

Method	Endogeneous 5-HIAA/ ($\mu\text{g/ml}$)	Recovery test of 5-HIAA		
		Added/ μg	Found/ μg	Recovery (%)
Manual	1.87	3.00	3.00	100
HPLC	1.55	3.00	3.06	102

Urine sample was collected for 24 h. Total volume of urine sample was 2450 ml.

Table 4 shows 5-HIAA content in a urine sample and recovery of 5-HIAA from the sample by manual and HPLC method. Excellent recoveries were observed for both manual and HPLC method. Content of 5-HIAA in urine measured by HPLC method was lower than that by the manual method. This might suggest that substances other than 5-HIAA interfere with the manual method and these substances can be eliminated by the HPLC method.

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