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FLUORIMETRIC DETERMINATION OF BIOGENIC 5-HYDROXY- AND 5-METHOXYINDOLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING PERCHLORIC ACID AS POST-COLUMN REAGENT

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SUMMARY

A method for the determination of biogenic 5-hydroxy- and 5-methoxyindoles using high-performance liquid chromatography with fluorescence detection was developed. The specific fluorescence of these indoles induced by perchloric acid was utilized as the post-column detection system. The determination limits were 3 pmol for 5-hydroxy-L-tryptophan and 5 pmol for serotonin (5-HT) and 5-hydroxyindole-3-acetic acid (HIAA). The method was applied to the determination of 5-HT and HIAA in rat brain tissues using 5-hydroxygramine as the internal standard.

INTRODUCTION

Many high-performance liquid chromatographic (HPLC) methods have been developed for the analysis of biogenic indole compounds owing to their physiological importance in neurochemistry and clinical chemistry[1–7]. In many of these HPLC methods, fluorescence detection[1–3,5,6] using native fluorescence based on the indole skeleton and electrochemical detection[4,7] have been used. However, as these detection methods are not always specific for biogenic indoles, the determinations have been often disturbed by co-existing biological substances in the samples and suitable pre-treatment such as organic solvent extraction[4] has been necessary before applying samples to these HPLC systems.

Recently, we have developed a method for the fluorimetric determination of biogenic indoles using perchloric acid (PCA)[8]. Indoles substituted at the 3-position gave specific fluorescence in PCA solution, the spectral characteristics of which are further separated into two types depending on whether the 5-position of the indole ring is substituted by a hydroxy (or methoxy) group or not; the former fluorescence has excitation and emission maxima at around 300 and 530 nm, respectively, and the latter at around 425 and 525 nm, respectively.

In this work, we attempted to apply the fluorogenic reaction to the specfic post-column detection of 5-hydroxy- and 5-methoxyindoles after HPLC separation and the method was applied to the determination of serotonin and 5-hydroxyindole-3-acetic acid in rat brain tissues.

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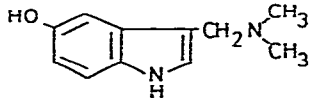
EXPERIMENTAL

Materials

5-Benzyloxygramine was purchased from Aldrich (Milwaukee, WI, U.S.A.). 5-Hydroxygramine (5-HG) hydrochloride was synthesized from 5-benzyloxygramine according to the method of Troxler *et al.*[9].



5-HG

Tryptamine hydrochloride and all 5-hydroxy- and 5-methoxyindoles were purchased from Sigma (St. Louis, MO, U.S.A.). L-Tryptophan and indole-3-acetic acid were obtained from Nakarai Chemicals (Kyoto, Japan). Perchloric acid (70%, w/v) was purchased from Kanto Chemical (Tokyo, Japan).

All other chemicals used were guaranteed reagents.

The acetate buffers were prepared by adjusting the pH of 20 m*M* sodium acetate solution with glacial acetic acid.

HPLC system

Fig. 1 shows the chromatographic system. Indoles were separated on a reversed-phase Finepak SIL C_{18}-10 column (10 μm, 250 × 4.6 mm I.D.; Japan Spectroscopic, Tokyo, Japan) at ambient temperature with either a mixture of 20 m*M* acetate buffer and methanol or a mixture of 20 m*M* acetate buffer and acetonitrile delivered by a double plunger type pump (Type KHU-W-52H; Kyowa Seimitsu, Tokyo, Japan). Samples were applied through a line sample injector (Type KLS-3T;

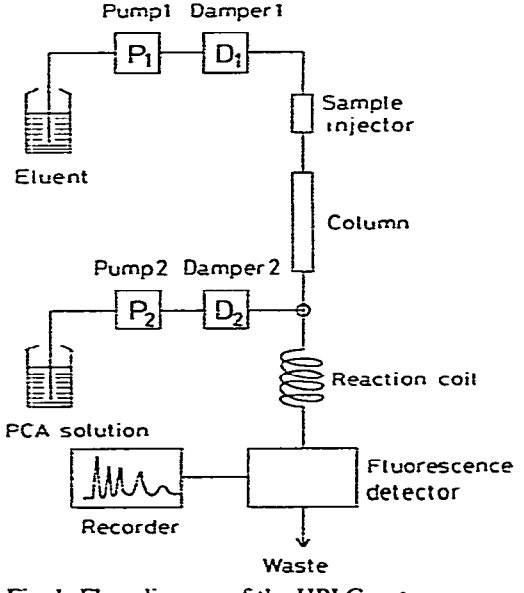


Fig. 1. Flow diagram of the HPLC system.

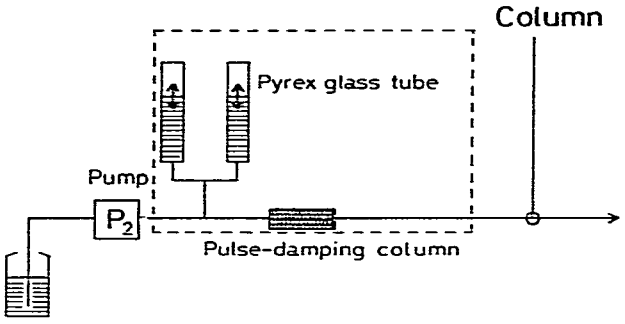


Fig. 2. Pulse-damping system for PCA solution. Two chromatographic tubes (500 × 8 mm I.D.) made of Pyrex glass with their top ends plugged were connected in parallel at their lower ends and placed between the pump and the pulse-damping Pyrex column (200 × 2 mm I.D.) packed with glass beads (200–400 mesh).

Kyowa Seimitsu) connected to a six-way valve (Type KHM-6V; Kyowa Seimitsu). A plunger damper (Type KD-300; Kyowa Seimitsu) was placed between the pump and the sample injector. Tubings placed before the separation column were made of stainless steel of 0.5 mm I.D., and other tubings were PTFE of 0.5 mm I.D. The column eluate was mixed in a three-way tee-piece (Type KYS-16; Kyowa Seimitsu) with 70% PCA delivered by a plunger type pump (Type KHU-26; Kyowa Seimitsu). To decrease the pulse of the flow of 70% PCA caused by the plunger pump, an air damper (Fig. 2) was used.

The mixture was introduced into the 80-μl flow cell by an Aminco Fluoro-Colorimeter (Type J4-7439; American Instrument Co., Silver Spring, MD, U.S.A.) equipped with a low-pressure mercury lamp coated with a fluorescent material (SPD-19B, λ_{max} = 312 nm; Toshiba, Tokyo, Japan). Coloured glass filters, UV-D33S (Toshiba) transmitting between 220 nm and 420 nm and Y-49 (Toshiba) transmitting above 480 nm, were used as the primary and secondary filters, respectively.

Native fluorescence was detected with a Shimadzu fluorescence detector (Type FLD-1; Shimadzu Seisakusho, Kyoto, Japan) equipped with a low-pressure mercury lamp (λ_{max} = 254 nm). A Shimadzu EX-1 filter (transmitting only around 254 nm) and a Shimadzu EM-1 filter (cutting off below 330 nm) were used as the primary and secondary filters, respectively.

Pre-treatment of rat brain tissues

Male Wistar rats weighing 220–230 g were used. Although the light and shade was not strictly controlled, all rats were killed at between 10.00 and 11.00 a.m. After removing brain tissues, about 100 mg of brain tissues were immediately mixed with 0.2 ml of 0.4 *M* PCA and 10 μl of 50 μM 5-HG (500 pmol), and homogenized with a Potter-Elvehjem homogenizer. After centrifugation at 15,000 *g* for 15 min, the supernatant was neutralized with 1 *M* potassium hydrogen carbonate solution. The resultant precipitate (potassium perchlorate) was removed by centrifugation and the supernatant was applied to the HPLC system. When a pineal gland (ca. 1 mg) was the sample, it was mixed with 0.1 ml of 0.4 *M* PCA and 5 μl of 50 μM 5-HG (250 pmol) and homogenized, then the same procedure used for other tissues was followed.

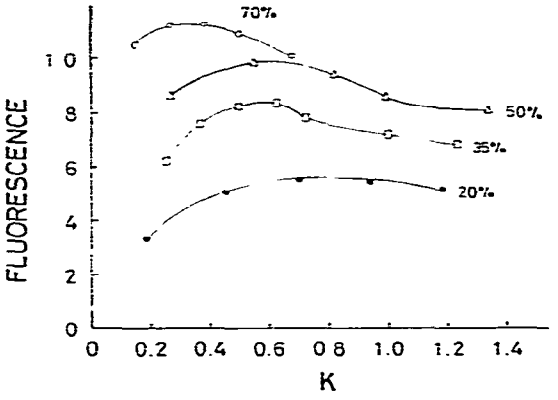


Fig. 3. Effect of the flow-rate ratio (K) of PCA to the eluate on the development of fluorescence from 5-HO-L-Trp. Elution was performed with a mixture of 20 mM acetate buffer (pH 4.1) and methanol (8:2) at a flow-rate of 0.98 ml/min. The concentration of PCA solution was varied from 20% to 70%. The reaction coil length was 10 m and the temperature was ambient.

RESULTS

Optimization for fluorescence detection

Using 5-hydroxy-L-tryptophan (5-HO-L-Trp), 5-hydroxytryptamine (5-HT), 5-hydroxyindole-3-acetic acid (HIAA), 5-methoxy-DL-tryptophan (5-MeO-DL-Trp) and N-acetylserotonin (NAS) as representatives of 5-hydroxy- and 5-methoxyindoles, the conditions for fluorescence detection were investigated.

First, the effect of the PCA concentration on the development of fluorescence was studied with 5-HO-L-Trp by mixing the various concentrations of the post-column PCA reagent with the eluate at various flow-rate ratios (K in Fig. 3). Similar results were obtained with other 5-hydroxy- and 5-methoxyindoles. From the data in Fig. 3, 70% was chosen as the concentration of PCA solution. As illustrated in Fig. 4,

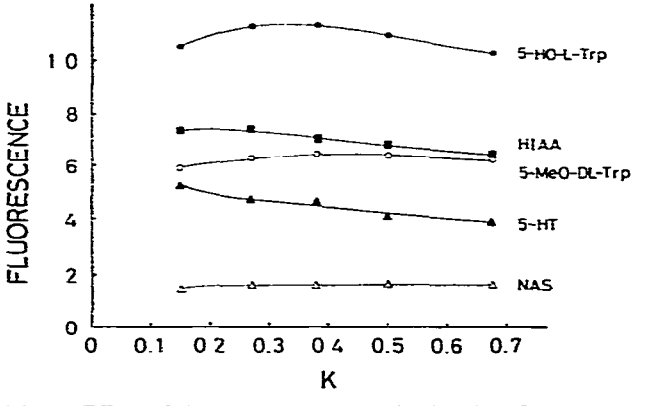


Fig. 4. Effect of the flow-rate ratio (K) of 70% PCA to the eluate on the development of fluorescence. Chromatographic conditions as in Fig. 3. Abbreviations: 5-HO-L-Trp = 5-hydroxy-L-tryptophan; 5-HT = 5-hydroxytryptamine; HIAA = 5-hydroxyindole-3-acetic acid; 5-MeO-DL-Trp = 5-methoxy-DL-tryptophan; NAS = N-acetylserotonin.

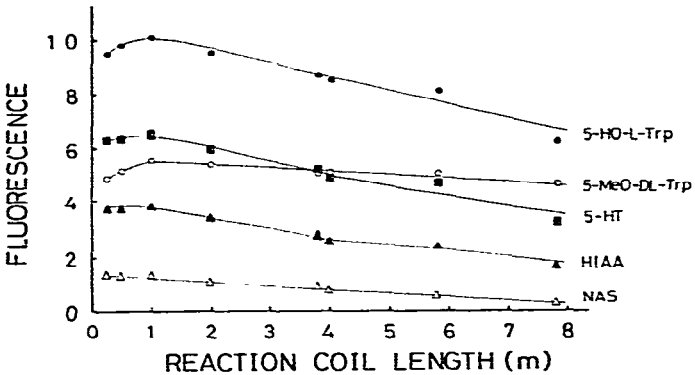


Fig. 5. Effect of the reaction time on the intensities of the PCA-induced fluorescence. Chromatographic conditions as in Fig. 3 except for reaction coil length; 70% PCA was mixed with the eluate at a flow-rate of 0.32 ml/min.

K did not have a marked influence on the fluorescence development with the five indoles tested. A *K* value of 0.33 was tentatively chosen.

Fig. 5 shows the relationship between the length of the reaction coil and the fluorescence intensity (peak height). All indoles gave the highest fluorescence intensities about 7 sec (reaction coil = 1 m) after mixing with 70% PCA.

Separation of biogenic indoles

The separation of biogenic indoles was successfully performed by stepwise elution with mixtures of 20 m*M* acetate buffer (pH 3.6) and methanol. The mixing ratio 8:2 was changed to 6:4 at 30 min (Fig. 6). Although L-tryptophan, tryptamine and indole-3-acetic acid were eluted in 13.5, 37.5 and 42 min, respectively, they were not

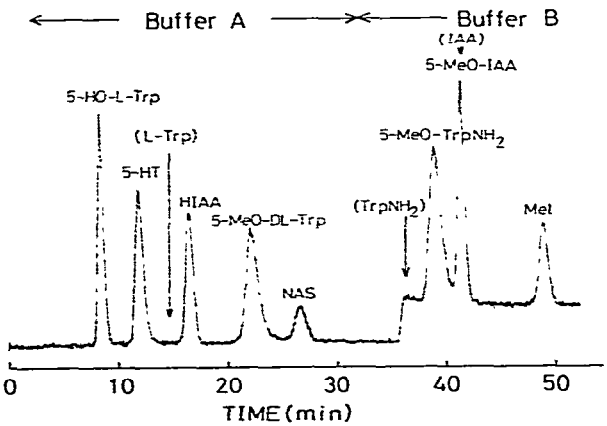


Fig. 6. Chromatogram of standard biogenic indoles; 50 pmol of each indole were injected. Buffer A, 20 m*M* acetate buffer (pH 3.6)–MeOH (8:2); buffer B, 20 m*M* acetate buffer (pH 3.6)–MeOH (6:4). Flow-rates: eluent, 0.75 ml/min; 70% PCA, 0.25 ml/min. Reaction coil length: 1 m. Abbreviations: L-Trp = L-tryptophan; $TrpNH_2$ = tryptamine; 5-MeO-$TrpNH_2$ = 5-methoxytryptamine; 5-MeO-IAA = 5-methoxyindole-3-acetic acid; Mel = melatonin; others as in Fig. 4.

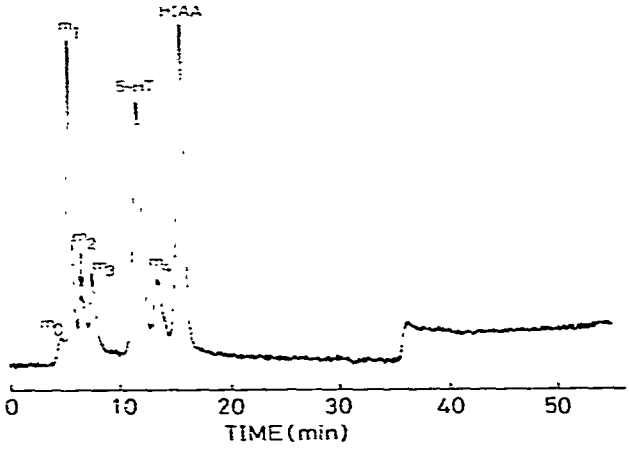


Fig. 7. Chromatogram obtained from rat brain. A 100-μl volume of sample solution was applied to the HPLC system. The peaks m_0, m_1, m_2, m_3 and m_4 were unidentified. Chromatographic conditions as in Fig. 6.

detectable under the present detection conditions owing to the lack of a hydroxy or methoxy group at the 5-position.

Sensitivity and reproducibility of the HPLC method

The calibration graphs were linear at concentrations up to 10 nmol and the determination limits were 3 pmol for 5-HO-L-Trp, 10 pmol for NAS and 5 pmol for 5-

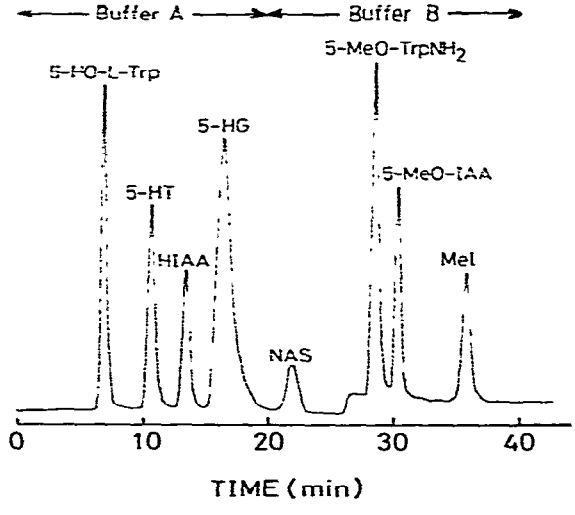


Fig. 8. Chromatogram of standard biogenic 5-hydroxy- and 5-methoxyindoles. Abbreviations: 5-HO-L-Trp = 5-hydroxy-L-tryptophan; 5-HT = serotonin; HIAA = 5-hydroxyindole-3-acetic acid; 5-HG = 5-hydroxygramine; NAS = N-acetylserotonin; 5-MeO-TrpNH$_2$ = 5-methoxytryptamine; 5-MeO-IAA = 5-methoxyindole-3-acetic acid; Mel = melatonin. The amounts injected were 250 pmol each except for 5-HG (500 pmol). Stepwise elution was performed with 20 m*M* acetate (pH 4.0)–acetonitrile (90:10, buffer A) and 20 m*M* acetate (pH 3.6)–acetonitrile (75:25, buffer B). The column temperature was maintained at 30°C.

HT, HIAA and 5-MeO-DL-Trp. The relative standard deviations were 1 % for 5-HO-L-Trp, 2 % for 5-HT, HIAA and 5-MeO-DL-Trp and 3 % for melatonin when 50 pmol of each indole compound were analysed five times.

Application to the analysis of rat brain tissues

A representative chromatogram obtained from rat brain (*ca.* 50 mg injected) is shown in Fig. 7. 5-HT and HIAA were detected together with unknown compounds (m_0–m_4), although other 5-hydroxy- or 5-methoxyindoles were not detected. When the same sample was monitored by the native fluorescence detection system, it was impossible to determine 5-HT and HIAA because many interfering peaks overlapped with the peaks of 5-HT and HIAA.

To increase the reliability of the determinations, the addition of an internal standard was examined, and the reults are shown in Fig. 8. Although the peak of 5-HG as the internal standard is overlapped by that of 5-MeO-DL-Trp, there seem to be no problems for the determination of 5-HT and HIAA because the amount of 5-MeO-L-Trp in rat brain sample is negligibly small, as shown in Fig. 7.

A whole rat brain was roughly separated into five parts and each was pretreated as described under Experimental. The amounts of biogenic indoles were determined by a standard additions extrapolation method by plotting peak heights of 5-HT and HIAA against amounts of indoles added to the brain samples. The reproducibility of the procedure for the determination of biogenic indoles was calculated. The relative standard deviations of the determined values of 5-HT and HIAA were 2 % and 6 %, respectively, when the same whole brain sample was analysed five times.

Fig. 9 shows the chromatogram obtained from whole brain. The values determined for 5-HT and HIAA (Table I) are in good agreement with previous values[7].

DISCUSSION

It is known that 5-HT fluoresces in hydrochloric acid solution[10,11] and this fluorescence has been utilized for the fluorimetric determination of 5-HT[12–17]. The

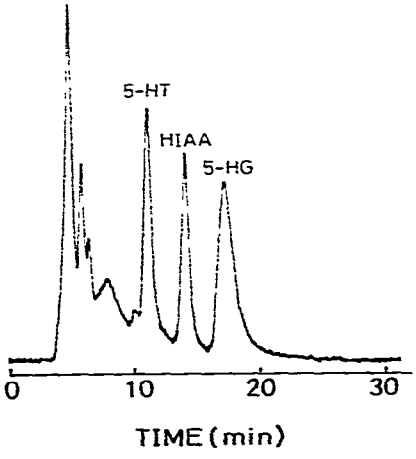


Fig. 9. Chromatogram obtained from rat whole brain. A 100-µl portion of the deproteinized sample (equivalent to *ca.* 30 mg of wet brain) was injected.

TABLE I

5-HT AND HIAA CONCENTRATIONS IN RAT BRAIN TISSUES

Tissue	*No. of analyses*	*5-HT (pmol/g ± S.D.)*	*HIAA (pmol/g ± S.D.)*
Cerebrum	6	1570 ± 540	2090 ± 510
Cerebellum	6	210 ± 60	480 ± 80
Thalamus, Hypothalamus Hypophysis	6	3080 ± 230	4640 ± 100
Pons, Medulla oblongata	6	3100 ± 820	3260 ± 960
Pineal gland	3	180 ± 60*	7 ± 3*

* pmol per pineal gland ± S.D.

fluorescing species induced by PCA might be the same as that induced by hydrochloric acid as the fluorescence characteristics in both acid solutions were the same; for example, the spectral characteristics closely resembled each other and both fluorescences were reversible in acidic and neutral solutions[8,18]. However, as the fluorescence induced by PCA was stronger than that induced by hydrochloric acid and PCA is less volatile than hydrochloric acid, PCA seems to be more suitable as the post-column reagent.

The present HPLC method permitted the specific detection of biogenic indoles with sensitivity comparable to that of conventional fluorescence detection methods[1-3,5,6] utilizing their native fluorescence. In the previous HPLC methods, the addition of internal standard to biological samples were not utilized, and the results were corrected by the recoveries of indoles from samples. However, these methods have the disadvantage of a poor reliability of determination as the recoveries varied with the sample tissues examined. In contrast, the present method using 5-HG as the internal standard seems to give a very reliable determination of biogenic indoles. Further, when the enrichment of minor indoles in samples is required for their determination, the use of an internal standard will be particularly useful.

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