Sensitive and Selective LC Determination of 5-Hydroxyindoles Through Online Electrochemical Fluorescence Derivatization Using Benzyamine

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Abstract
In this paper we describe a highly sensitive and selective LC method for the determination of 5-hydroxyindoles (serotonin, N-acetylserotonin, 5-hydroxyindole-3-acetamide, 5-hydroxytryptophan, 5-hydroxyindole-3-acetic acid, and 5-hydroxytryptophol) through post-column electrochemical derivatization and fluorescence detection. These 5-hydroxyindoles can be separated within 40 min by reversed-phase liquid chromatography using 250 mM acetate buffer/acetonitrile [pH 6.5; 95:5 (v/v)] under conditions of isocratic elution; then they were subjected to electrochemical oxidation with benzyamine to produce their corresponding fluorescent derivatives. We detected these derivatives spectrofluorometrically at 480 nm upon excitation at 345 nm. The detection limits (S/N = 3) of these 5-hydroxyindoles were in the range 3.2–180 fmol per 20-µL injection.

Keywords: Serotonin; 5-Hydroxyindole; Derivatization; Electrolytic oxidation; Fluorescence detection; Benzyamine

1. Introduction
5-Hydroxytryptamine (serotonin; 5-HT), one of biologically occurring 5-hydroxyindoles (5-HIs), also presents in the central nervous system and blood platelets. Because intestinal carcinoid tumors release large amounts of 5-HT, higher concentrations of 5-hydroxyindole-3-acetic acid (5-HIAA), the major metabolite of 5-HT, are found in the urine of such patients. Increased levels of 5-HT also have been implicated in a number of mental disorders, including depression [1-3], migraine [4,5], and schizophrenia [6-8]. Therefore, the ability to simultaneously determine 5-HT, its precursor, 5-hydroxytryptophan, and its metabolites [5-HIAA, N-acetylserotonin (NAS), 5-hydroxyindole-3-acetamide (5-HA), 5-hydroxytryptophan (5-HTP), and 5-hydroxytryptophol (5-HTOL)] in biological fluids would be useful for neuroscience and pharmacology investigations and for medical diagnosis of the various disorders.

A number of methods have been developed that use LC for the determination of 5-HIs and taking advantage of their native fluorescence [9-12] or through electrochemical detection [13-15]. These detection methods, however, are not always specific for 5-HIs. Therefore, suitable pre-treatment processes, such as liquid–liquid or liquid–solid phase extractions, are often required to avoid any interference from biological samples.

Recently, direct measurement of 5-HIs using LC mass spectrometry is also common, but it is necessary to consider the fluctuation of peak intensity due to the influence of biological matrix and the stability of retention in LC [16].

In a previous study, we found that several aromatic methylamines, such as benzyamine and...
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3,4-dimethoxybenzylamine, react selectively with 5-HIs in a weakly alkaline medium in the presence of potassium hexacyanoferrate(III); these derivatives are intensely fluorescent in alkaline media [17]. This method is so selective for 5-HIs that we have applied it to post-column fluorescence derivatization LC for the determination of 5-HIs in human plasma [18], in human urine [19] and in microdialysis samples taken from rats [20-22] and mice [23,24]. Unfortunately, the potassium hexacyanoferrate(III) solution, which is employed only as a chemical oxidizing agent, is harmful and requires preparation just prior to its use because of its instability. In addition, it is subject to absorption on an inner wall of the flow-line or flow-cell of the fluorescence detector, and its accumulation greatly increases the background noise and, consequently, decreases the sensitivity of detecting the 5-HIs. Therefore, frequent washing of the flow-line and flow-cell is necessary to retain high sensitivity during analyses.

In this study, we adopted an online electrochemical oxidation rather than conventional chemical oxidation for the fluorescence derivatization of the 5-HIs with benzylamine. The 5-HIs were separated through reversed-phase LC under isocratic elution and then mixed effluent with a reagent solution including benzylamine. This mixed solution was oxidized in electrochemical cells to produce the corresponding highly fluorescent derivatives (Fig. 1). The conditions of the LC separation, the electrochemical oxidation, and the fluorescence derivatization reaction were optimized to allow the sensitive and selective determination of 5-HIs. Furthermore, the reactivities of 5-methoxyindoles, other indoles, catecholamines, and their metabolites in this new system were also examined.

Fig. 1. Fluorescence derivatization of 5-HIs through electrochemical oxidation in the presence of benzylamine.

2. Experimental

2.1. Reagents and solutions

Deionized and distilled water, purified using a Milli-QII (Merck Millipore, Milford, MA, USA) system, was used to prepare all of the aqueous solutions. 5-HT, NAS, 5-HA, 5-HTP, 5-HIAA, and 5-HTOL were purchased from Sigma-Aldrich (St. Louis, MO, USA); standard solutions (1 mM) were prepared in water, stored at -20°C, and diluted further with water to the desired concentrations immediately prior to use. Benzylamine was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals were obtained at the highest purity available and used as received. All mobile phase solutions were used after filtration using a Millipore-LH (pore size: 0.45 μm; PTFE, Merck Millipore).

2.2. LC-fluorescence detection system and conditions

Figure 2 presents a schematic flow diagram of the LC system. Isocratic elution was performed using a PU-980 liquid chromatograph pump (Jasco, Tokyo, Japan) equipped with a Rheodyne (Cotati, CA, USA) Model 7125 syringe-loading sample injector valve (20-μL loop). The analytical column used was a CAPCELL PAK C18MG (150 × 4.6 mm I.D.; particle size: 5 µm; Shiseido, Tokyo, Japan). The column was maintained at ambient temperature (22 ± 4°C). The guard column used was a TSK guardgel ODS-80TM (15 × 3.2 mm I.D.; Tosoh, Tokyo, Japan). The mobile phase was 250 mM acetate buffer (pH 6.5)/acetonitrile [95:5 (v/v)], and the flow rate was 0.9 mL/min. A Coulochem II coulometric monitor (ESA Associates, Bedford, MA, USA) was employed for the electrochemical derivatization of the 5-HIs. This system was equipped with Model 5010 double detector cells (porous graphite) for the electrochemical oxidation and reduction. The working electrode material of each electrochemical cell was porous graphite; Pt and Pt/H2 were the counter and reference electrodes, respectively. A pulsed potential was applied to the first electrochemical cell, with the time sequence of potentials set as follows: time = 0.00 sec, potential = +0.30 V; time = 0.10 sec, potential = -1.0 V; time = 0.15 sec, potential = +0.30 V; time = 0.2 sec. The potential of the second electrochemical cell was maintained at +0.30 V. The effluent from the LC column was then mixed with a stream of reagent solution, a mixture of acetonitrile, 25 mM sodium tetraborate, and 125 mM sodium hydroxide [2:1:1 (v/v)] containing 20 mM benzylamine; it was delivered using an L-6000 pump (Hitachi, Tokyo, Japan) at a flow rate of 0.45 mL/min. The fluorescence derivatization reaction was performed in the electrochemical cells after passing a heating coil (PEEK tubing, 7 m × 0.5 mm I.D.) immersed in a CTO-6A column oven (Shimadzu, Kyoto, Japan) at a temperature of 100°C. After fluorescence derivatization, the eluent was passed through a cooling coil (PEEK tubing, 0.5 m × 0.5 mm I.D.). The coil was inserted to obtain stable fluorescence by lowering the high temperature during the reaction to room temperature. The resulting fluorescence was monitored at
an excitation wavelength of 345 nm and an emission wavelength of 480 nm by using an L-7480 spectrofluorometer (Hitachi) equipped with a 12-µL flow cell. Chromatograms were recorded using a D-7500 Chromato-Integrator (Hitachi).

Fig. 2. Schematic flow diagram of the post-column LC-fluorescence detection system used for the determination of 5-HIs. P₁ and P₂, LC pumps; I, injection valve (20 µL); G, guard column; M, mixing device; HC, heating coil; EC₁ and EC₂, electrochemical cells; CC, cooling coil; ER, electrochemical regulator (coulometer); FD, fluorescence detector; Rec, integrator; E, eluent; R, reagent solution.

3. Results and discussion

3.1. LC separation of 5-HIs

Figure 3 presents a typical chromatogram obtained using a standard mixture of six 5-HIs. We achieved good separation of all the 5-HI derivatives, except for those of 5-HTP and 5-HIAA, within 40 min on the ODS column when performing isocratic elution with 250 mM acetate buffer/acetonitrile [pH 6.5; 95:5 (v/v)] as the mobile phase; the retention times were 5.0 (5-HTP), 5.1 (5-HIAA), 9.3 (5-HT), 11.3 (5-HA), 22.5 (5-HTOL), and 36.5 min (NAS), respectively.

We investigated the effect of mobile phase pH on the chromatogram in the pH range of 5.0–7.0. The fluorescence peak heights for all of the 5-HIs, except for that of NAS, reached their maxima at pH 6.5. Therefore, a buffer at pH 6.5 was used for all further experiments.

3.2. Online electrochemical derivatization

Two types of electrochemical detector, amperometric and coulometric, have been utilized in the LC analysis of 5-HIs. In this study, we wanted to perform quantitative oxidation of the 5-HIs with benzylamine to produce their fluorescent derivatives and, hence, we employed coulometric detection.

The fluorescence derivatization reaction proceeds through electrochemical oxidation at a constant voltage. Unfortunately, the peaks of some of the 5-HIs tailed to a great extent, and an increase in cell pressure was also observed, presumably because of irreversible adsorption of the 5-HIs or their fluorescent derivatives onto the cells.

|           | 1st cell                  | 2nd cell                  |
|-----------|---------------------------|---------------------------|
| (a)       | +0.30 V 0.1 s             | +0.30 V 0.1 s             |
| (b)       | +1.0 V 0.15 s             | 0 V                       |
| (c)       | +0.30 V 0.1 s             | +0.30 V 0.1 s             |
| (d)       | 0 V                       | -1.0 V 0.15 s             |
| (e)       | +0.30 V                   | +0.30 V                   |

Fig. 3. Chromatogram obtained using a standard mixture of 5-HIs. A portion (20 µL) of a standard solution (20 pmol each on column) was applied to the LC system. Peaks: (1) 5-HTP and 5-HIAA; (2) 5-HT; (3) 5-HA; (4) 5-HTOL; (5) NAS.

Fig. 4. (A) Voltage patterns applied to the electrochemical cells and (B) their corresponding chromatograms. For peak labels, see Fig. 3.
To prevent such adsorption, we introduced a pulse mode—a continuously changing cell voltage—to the determination voltage (+0.30 V), washing voltage (-1.0 V), and conditioning voltage (+0.30 V). This mode provides very high signal response reproducibility along with the cleaning and reactivation of the electrode surface. The two electrochemical cells had the ability to perform the pulse mode built-in to their cells, but the pulsed voltage can be applied to either of them. We investigated the effects that applying five different voltage patterns to the electrochemical cells [Fig. 4(A)] has on their chromatograms [Fig. 4(B)]. We observed sharp peaks in pattern (e), in which a constant voltage was applied, but the peak height continued to decrease and the cell pressure increased considerably upon repeated sample injection. In patterns (b), (c), and (d), a remarkable degree of reduction in peak heights and increased peak tailings were observed. In pattern (a), the cell pressure increases were much smaller than those of the other patterns, and peak tailing was also suppressed. Therefore, pattern (a) for use in subsequent electrochemical oxidations was selected.

The addition of electrolyte into the mobile phase also accelerated the efficiency of the electrochemical oxidation. By varying the acetate concentration in the range 50–350 mM, the fluorescence peak heights reached their maximum values at 250 mM and thereafter remained constant, but the intensities of background signals increased at concentrations > 300 mM. When other electrolytes, such as potassium chloride, were added to a mobile phase in an effort to raise the electrochemical oxidation efficiency, no apparent effect on the peak height was observed but increased background noise did occur; thus, a 250 mM acetate concentration was utilized for the subsequent experiments.

Figure 5 displays the effects that the benzylamine and sodium hydroxide concentrations and the heating coil temperature have on the derivatization reactions. Benzylamine concentrations greater than 20 mM in the reagent solution gave the best peak heights for all of the examined 5-HIs, except for 5-HIAA [Fig. 5(A)]. Because the maximum peak heights were almost attained at 20 mM, and concentrations above 20 mM caused an increase in the intensities of background signals, 20 mM benzylamine was selected for all subsequent procedures. Fig. 5(B) presents the effect of the sodium hydroxide concentration in the reagent solution, varied in the range 25–200 mM. The maximum peak height was obtained when using 125 mM NaOH for 5-HTOL, NAS and 5-HT and 150 mM NaOH for the other 5-HIs. To detect both 5-HT and 5-HIAA with high sensitivity, we took 150 mM to be the optimal concentration of NaOH. At this concentration, the pH of the final reaction mixture was ca. 10.0. In our previous fluorescence derivatization reaction of 5-HIs using the oxidizing agent, we found that the optimized heating coil length and temperature were 7 m (0.5 mm I.D.) and 100°C, respectively [24]. In our present approach, no derivatization reaction occurs in the heating coil; thus, we fixed the coil length at 5 m and investigated the effect that the coil temperature has on the fluorescence derivatization [Fig. 5(C)].

![Fig. 5.](image-url) Effects that the (A) benzylamine and (B) sodium hydroxide concentrations in the reagent solution and (C) the heating coil temperature have on the fluorescence derivatization of 5-HIs. (1) 5-HTOL; (2) 5-HA; (3) NAS; (4) 5-HT; (5) 5-HIAA; (6) 5-HTP.
The reaction rate increased upon increasing the temperature, but the upper limit of the coil heater temperature was 100°C. From these results, the following conditions were selected for future experiments: 20 mM benzylamine, 150 mM sodium hydroxide, and a coil temperature of 100°C.

3.3. Calibration graphs, detection limits, and precision of the method

The relationships between the peak areas and the amounts of the individual 5-HIs were linear over concentration ranges from 0.2–20 pmol per 20-µL injection volume; the linear correlation coefficients were greater than 0.989 (n = 6) for all of the 5-HIs we studied. The intra-day precision was established from repeated determinations (n = 6) using a standard mixture of the 5-HIs (1 µM). The relative standard deviations of peak area were 3.7 (5-HT), 9.3 (NAS), 4.7 (5-HA), 180 (5-HTP), 21 (5-HIAA), and 19 (5-HTOL).

Table 1. Detection limits (DL) and retention times (tR) for 5-HIs, 5-methoxyindoles, other indoles, catecholamines, and their metabolites.

| Compound                      | DL (fpmol) | tR (min) |
|-------------------------------|------------|----------|
| 5-Hydroxyindoles              |            |          |
| 5-Hydroxytryptamine (5-HT)    | 14         | 9.3      |
| N-acetylsertotonin (NAS)      | 9.3        | 36.5     |
| 5-Hydroxyindole-3-acetamide (5-HA) | 4.7         | 11.3     |
| 5-Hydroxytryptophan (5-HTP)   | 180        | 5.0      |
| 5-Hydroxyindole-3-acetic acid (5-HIAA) | 21         | 5.1      |
| 5-Hydroxytryptophol (5-HTOL)  | 3.2        | 22.5     |
| 5-Hydroxyindole               | 19         | 27.7     |
| 5-Methoxyindoles              |            |          |
| 5-Methoxytryptamine (5-MT)    | N.D.       |          |
| Melatonin (Mel)               | N.D.       |          |
| 5-Methoxytryptophan (5-MTP)   | N.D.       |          |
| 5-Methoxyindole-3-acetic acid (5-MIAA) | N.D.   |          |
| 5-Methoxytryptophol (5-MTOL)  | N.D.       |          |
| Other indoles                 |            |          |
| Tryptamine                    | N.D.       |          |
| Tryptophan                    | N.D.       |          |
| Indole-3-acetic acid          | N.D.       |          |
| Tryptophol                    | N.D.       |          |
| 4-Hydroxyindole               | N.D.       |          |
| Catecholamines and their metabolites | |      |
| Dopamine                      | 1700       | 5.9      |
| Epinephrine                   | 83         | 4.1      |
| Norepinephrine                | 530        | 4.9      |
| 3,4-Dihydroxy-L-phenylalanine (L-DOPA) | 790       | 4.6      |
| Methanephrine                 | 2300       | 6.1      |
| Normetanephrine               | 6200       | 4.8      |
| 3-Methoxytryptamine           | 1400       | 10.2     |
| 3,4-Dihydroxyxymandelic acid (DOMA) | 980         | 3.1      |
| 3,4-Dihydroxyphenylacetic acid (DOPAC) | 510      | 3.9      |
| 3,4-Dihydroxyphenyl glycol (DOPEN) | 350        | 4.8      |
| Vanilmandelic acid (VMA)      | 5400       | 3.5      |
| Homovanilic acid (HVA)        | 760        | 5.5      |
| 4-Hydroxy-3-methoxyphenyl glycol (MOPEG) | 2800   | 7.3      |

Table 1 lists the retention times and detection limits for the nine catecholamine metabolites were also investigated. The reactivity of this system toward five 5-methoxyindoles, five indoles, four catecholamines, and nine catecholamine metabolites were also investigated. The detection limits (signal-to-noise ratio = 3) were 14 (5-HT), 9.3 (NAS), 4.7 (5-HA), 180 (5-HTP), 21 (5-HIAA), and 3.2 fmol (5-HTOL) per 20-µL injection. These values, except for that of 5-HTP, are all ca. 13–94 times lower than those we reported previously when using the oxidizing-agent-based post-column derivatization method [19]; the detection limit for 5-HTP was about the same as that we obtained previously.

3.4. Reactivity toward 5-methoxyindoles, other indoles, catecholamines, and their metabolites

The reactivity of this system toward five 5-methoxyindoles, five indoles, four catecholamines, and nine catecholamine metabolites was established from repeated determinations (n = 6) using a standard mixture of the 5-HIs (1 µM). The relative standard deviations of peak area were 3.7 (5-HT), 9.3 (NAS), 4.7 (5-HA), 180 (5-HTP), 21 (5-HIAA), and 19 (5-HTOL).

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3.4. Reactivity toward 5-methoxyindoles, other indoles, catecholamines, and their metabolites

The reactivity of this system toward five 5-methoxyindoles, five indoles, four catecholamines, and nine catecholamine metabolites were also investigated. Table 1 lists the retention times and detection limits for the benzylamine derivatives. The 5-methoxyindoles and other indoles examined did not result in any peaks appearing in the chromatograms. All of the examined catecholamines, except for epinephrine, and their metabolites resulted in very small peaks appearing in the chromatograms; epinephrine appeared as a relatively large peak in the chromatogram. These tendencies are attributed to the relative reactivities of the analytes toward benzylamine; indeed, they correlate well with the results we obtained when using the chemical oxidation method [25].

None of the following biological compounds and pharmaceutical drugs, each at a concentration of 10 µM, afforded any peaks under the present conditions, apart from their native fluorescent peaks: all the essential 1-amino acids, nucleic acid-related compounds (adenine, guanine, thymine, cytosine, uracil, adenosine, guanosine, tymidine, cytidine, uridine), sugars (D-glucose, D-fructose, D-galactose, D-ribose, D-xyllose, D-mannose, N-acetyl-D-glucosamine), vitamins (B1, B2, B6, C), and other compounds (nicotinic acid, nicotinamide, tranexamic acid, phenethylamine, hydralazine hydrochloride,isoniazid). These observations suggest that our proposed method is highly selective for 5-HIs.

4. Conclusion

In conclusion, we have developed a highly sensitive analytical method for 5-HIs that does not require the addition of harmful chemical oxidizing agent. Online oxidation of the analytes and benzylamine leads to the formation of fluorescent derivatives. The peak tailing and electrolytic cell-pressure increase when using constant applying voltage were greatly improved by applying pulse voltage. The detection limits for 5-HIs of this method were femto-mol level and were almost same as those obtained from the chemical oxidizing method. Tested biological substances except 5-HIs and some catecholamines did not afford any peaks on the chromatograms in this method. Although further chromatographic separation of 5-HTP and
5-HIAA is required for simultaneous analysis of 5-HIs, these high sensitivity and selectivity permit the direct use of diluted real sample without tedious clean-up procedure. Our results obtained suggests that the present method may be applicable to biological investigations such as microdialysis and medical diagnosis, similar to the high-sensitivity fluorescence derivatization analysis method of 5-HIs that we have achieved by the chemical oxidation method.

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