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3,4-Dimethoxybenzylamine as a sensitive pre-column fluorescence derivatization reagent for the determination of serotonin in human platelet-poor plasma

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Abstract

3,4-Dimethoxybenzylamine is shown to be a highly sensitive pre-column fluorescence derivatization reagent for the determination of serotonin in plasma by high-performance liquid chromatography. The reagent reacts selectively with 5-hydroxyindoles including serotonin in slightly alkaline media in the presence of potassium hexacyanoferrate(III) to give highly fluorescent derivatives. The derivatives of six standard 5-hydroxyindoles (5-hydroxytryptophan, 5-hydroxyindole-3-acetic acid, serotonin, 5-hydroxyindole-3-acetamide, *N*-acetyl-5-hydroxytryptamine and 5-hydroxytryptophol) are separated within 18 min by isocratic elution using acetonitrile–10 m*M* phosphate buffer (pH 6.0)–50 m*M* 1-hexanesulfonic acid on a Wakosil II 5C18RS reversed-phase column. The detection limits (signal-to-noise ratio=3) for the indoles were 1.0–5.7 fmol in a 100-μl injection volume. The method was applied to the measurement of serotonin in human platelet-poor plasma.

Keywords: 3,4-Dimethoxybenzylamine; Serotonin

1. Introduction

Serotonin is well known to act as a neurotransmitter in the control and regulation of many brain functions, and has been strongly implicated in several pathological conditions such as essential hypertension [1,2], migraine [3], depression [4] and carcinoid syndrome [5]. Abnormal concentrations of serotonin in plasma have been shown to reflect the serotonergic function in the central nervous system.

Several methods including high-performance liquid chromatography (HPLC) with native fluorimetric [6–8] and electrochemical detection [9–14], gas chromatography–mass spectrometry [15], radioimmunological [16–18] and radioenzymatic [19,20] techniques have been described for the determination of serotonin and/or the related compounds in body fluids.

In the previous work [21], we have reported that aromatic methylamines react selectively and sensitively with 5-hydroxyindoles in weakly alkaline media in the presence of potassium hexacyanoferrate(III). Benzylamine was found to be a suitable post-column derivatization reagent due to its quite low background fluorescence [22]. However, a more sensitive method is required for the determination of serotonin in biological samples, in which the amine is present at an extremely low concentration.

The purpose of this study is to find a more sensitive reagent for pre-column derivatization of

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serotonin. Of thirteen aromatic methylamines tested, 3,4-dimethoxybenzylamine was found to be the most sensitive as a pre-column fluorescence derivatization reagent for serotonin. Hence, we have developed a sensitive and selective fluorimetric HPLC method with pre-column derivatization using 3,4-dimethoxybenzylamine for the determination of serotonin in human platelet poor plasma with simple pre-treatment. 5-Hydroxyindole-3-acetamide was used as an internal standard (I.S.).

2. Experimental

2.1. Chemicals and solutions

De-ionized and distilled water, purified with a Milli-Q II (Millipore, Milford, MA, USA) system, was used for all aqueous solutions. Serotonin and its related compounds were purchased from Sigma (St. Louis, MO, USA). 3,4-Dimethoxybenzylamine hydrochloride was used after purification by recrystallization with ethanol. Other chemicals were of the highest purity available and were used as received. The 20 m*M* solutions of 3,4-dimethoxybenzylamine and the other aromatic methylamines were prepared by dissolving the reagents in a mixture of dimethylsulfoxide and water (1:1, v/v); the reagent solutions were stable for at least 1 week, even at room temperature. Potassium hexacyanoferrate(III) solution (30 m*M*) was prepared in a mixture of dimethylsulfoxide and water (4:1, v/v); the solution was stable for at least 1 day. Standard solutions of 5-hydroxyindoles were prepared in water and kept frozen (−20°C) in amber test tubes.

2.2. Chromatography

A Hitachi L-6000 high-performance liquid chromatograph (Tokyo, Japan) equipped with a high-pressure sample injector (100-μl loop) and a Jasco FP-920 Fluorescence Spectromonitor (Tokyo, Japan) fitted with a 16-μl flow-cell was used. It was operated at an excitation wavelength of 345 nm and an emission wavelength of 475 nm. The separations were performed on a Wakosil-II 5C18 RS reversed-phase column (150×4.6 mm I.D.; particle size, 5 μm; Wako Pure Chemicals, Tokyo, Japan), with isocratic elution with a mixture of acetonitrile, 10 m*M* phosphate buffer (pH 6.0) and 50 m*M* 1-hexanesulfonic acid sodium salt (7:9:4, v/v) at a flow-rate of 0.8 ml/min. The column temperature was ambient (20–23°C).

2.3. Fluorescence derivatization

To a 100-μl portion of a test solution of 5-hydroxyindoles were added 50 μl of 100 m*M* tris hydrochloric acid buffer (pH 9.0), 100 μl of 20 m*M* 3,4-dimethoxybenzylamine (or the other aromatic methylamines) solution and 50 μl of 30 m*M* potassium hexacyanoferrate(III) solution, successively. The mixture was allowed to stand at room temperature for approx. 2 min. A 100-μl portion of the final reaction mixture was injected into the chromatograph. To prepare the reagent blank, 100 μl of water in place of 100 μl of test solution was carried out through the procedure.

2.4. Human platelet-poor plasma sample

Human platelet poor plasma samples were prepared in the usual manner [9,10]. Most circulating serotonin exists in blood platelets. Therefore, platelets were separated from whole blood to eliminate the possibility of contamination of plasma from this source. Venous blood was collected into a plastic test tube containing Na_2EDTA (1 mg Na_2EDTA/ml of blood). After gentle agitation, the mixture was centrifuged at 1500 *g* for 30 min at 4°C. The resulting human platelet poor plasma was stored at −20°C until required for assay.

2.5. Determination of serotonin in human platelet-poor plasma

Human platelet-poor plasma was obtained from healthy volunteers.

For the preparation of a plasma sample, a 40-μl aliquot of human platelet-poor plasma, 20-μl of 20 n*M* 5-hydroxyindole-3-acetamide (I.S.) and 140-μl of 100 m*M* Tris hydrochloric acid buffer (pH 9.0) were pipetted into a Ultracent-30 cartridge (cellulose, 92×15 mm O.D., Tosoh, Tokyo, Japan), successively. The cartridge was centrifuged at 1500 *g* for 30

min. The filtrate (100 μl) from the cartridge was treated according to the fluorescence derivatization.

For the establishment of the calibration graph, 20 μl of the 5-hydroxyindole-3-acetamide (I.S.) solution was replaced by an I.S. solution containing serotonin (20 fmol–10 pmol/20 μl). The net peak height ratios of the serotonin and I.S. were plotted against the concentrations of serotonin added.

3. Results and discussion

3.1. Selection of 3,4-dimethoxybenzylamine as a pre-column derivatization reagent for serotonin

In a previous work [21], eight aromatic methylamines were screened as fluorogenic reagents for 5-hydroxyindoles by a manual method. In this work, thirteen aromatic methylamines shown in Table 1 were studied as pre-column fluorescence derivatization reagents in HPLC using serotonin as a representative 5-hydroxyindole of biological importance. The fluorescence intensities (peak height) and retention times of the derivatives are shown in Table 1. 3,4-, 2,3- and 3,5-dimethoxybenzylamines provided the most intense fluorescence; 3,4-dimethoxybenzylamine hydrochloride was tentatively selected.

Table 1
Relative fluorescence intensities (RFI) and their retention times (t_R) of the derivatives produced by the reaction of serotonin with aromatic methylamines

Aromatic methylamine	t_R (min)	RFI[a]
3,4-Dimethoxybenzylamine	9.2	100.0
2,3-Dimethoxybenzylamine	10.9	100.0
3,5-Dimethoxybenzylamine	17.6	98.0
Benzylamine	15.5	37.9
4-Methoxybenzylamine	16.3	50.6
4-Methylbenzylamine	27.2	28.1
3-Methylbenzylamine	30.1	24.3
4-Hydroxy-3-methoxybenzylamine	5.9	13.6
4-Aminomethylbenzoic acid	5.2	5.2
2-Aminomethylpyridine	5.2	9.2
3-Aminomethylpyridine	5.2	9.2
1-Naphthalenemethylamine	56.0	4.6
4-Dimethylaminobenzylamine	18.8	30.4

[a] The fluorescence intensity (peak height) obtained by the reaction of serotonin with 3,4-dimethoxybenzylamine was taken as 100.0.

3.2. HPLC and derivatization conditions

Fig. 1 shows a typical chromatogram obtained with a standard mixture of the indoles tested; their retention times (min) and relative standard deviations (%, $n=4$, in parentheses) were 4.4 (1.2) (5-hydroxyindole-3-acetic acid), 4.4 (1.0) (5-hydroxytryptophan), 9.2 (1.2) (serotonin), 13.5 (1.1) (5-hydroxyindole-3-acetamide), 16.0 (1.3) (*N*-acetyl-5-hydroxytryptamine) and 17.5 (1.4) (5-hydroxytryptophol). The fluorescence excitation and emission maxima of the indoles were around 345 and 475 nm, respectively, irrespective of the indoles tested.

The conditions for the fluorescence derivatization of 5-hydroxytryptophan with 3,4-dimethoxybenzylamine were described previously by the manual method [21]. In this study, the conditions for six 5-hydroxyindoles were examined using HPLC. The

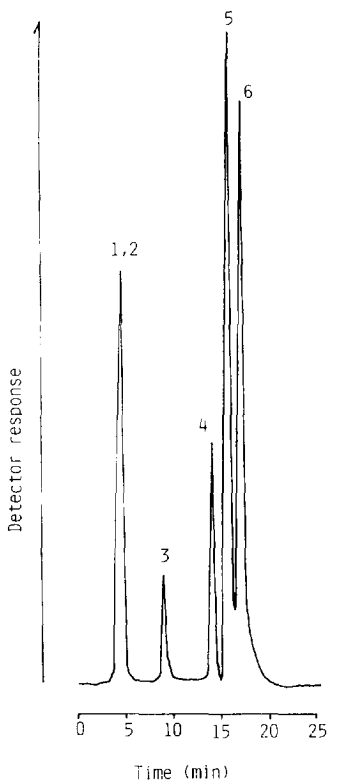

Fig. 1. Chromatogram of a standard mixture of six 5-hydroxyindoles (3.3 pmol/injection). Peaks: 1=5-hydroxytryptophan; 2=5-hydroxyindole-3-acetic acid; 3=serotonin; 4=5-hydroxyindole-3-acetamide; 5=*N*-acetyl-5-hydroxytryptamine; 6=5-hydroxytryptophol.

resulting optimal conditions were similar to those for 5-hydroxytryptophan [21] except for potassium hexacyanoferrate(III) concentration. Maximum fluorescent peak for 5-hydroxytryptophan was obtained with potassium hexacyanoferrate(III) at concentrations of 10–15 m*M*. Conversely, those for the 5-hydroxyindoles including serotonin gave maxima in the range of 20–40 m*M*; a 30 m*M* potassium hexacyanoferrate(III) solution was adopted in the recommended procedure (Fig. 2).

The fluorescent derivatives in the reaction mixture were stable for at least 15 h at room temperature.

3.3. Validation of the method

The relationships between the peak heights and the amounts of the individual indoles were linear up to at least 100 pmol per injection volume (100 μl). The detection limits (fmol, signal-to-noise ratio=3) were 2.9 (5-hydroxytryptophan), 3.0 (5-hydroxyindole-3-acetic acid), 5.7 (serotonin), 2.8 (5-hydroxyindole-3-acetamide), 1.0 (*N*-acetyl-5-hydroxytryptamine) and 1.1 (5-hydroxytryptophol) for an injection volume of 100 μl. The precision was established by repeated determinations of a standard mixture of the indoles (1.0 nmol each/ml). The relative standard deviations did not exceed 2.9% for all the indoles examined ($n=10$ in each case).

3.4. Determination of serotonin in human platelet-poor plasma

Plasma should be deproteinized, otherwise the column packing for HPLC is considerably damaged. The deproteinization could be done by ultrafiltration. The use of perchloric acid or trichloroacetic acid for the deproteinization of plasma in the usual manner caused low derivatization reaction yields of serotonin (approx. 50%). One of the reasons for the low yields in both cases might be due to the difficulties in adjusting the pH values to the optimum (pH 9.0) for the derivatization.

A typical chromatogram obtained with platelet-poor plasma from a healthy subject is shown in Fig. 3. Peaks 3 and 4 correspond to serotonin and I.S., respectively. Peaks were attributed on the basis of retention times in comparison with the standard, and also co-chromatography of the standard and a plasma sample using 20–40% (v/v) acetonitrile as mobile phase. When the analysis was performed without

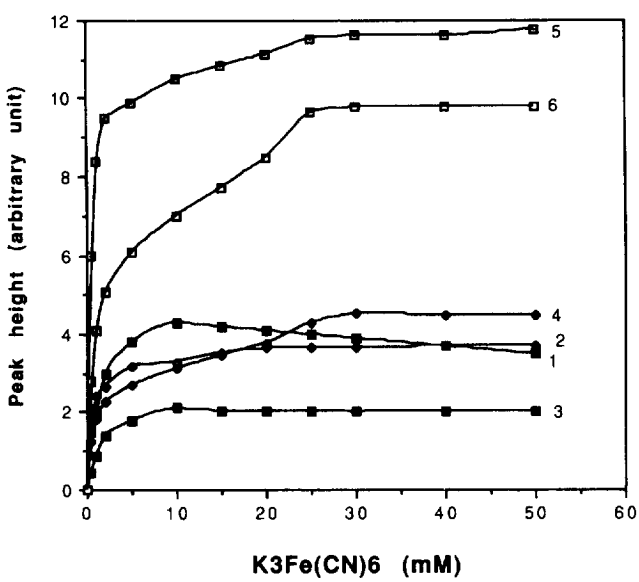

Fig. 2. Effect of potassium hexacyanoferrate(III) concentration on the fluorescence derivatization of 5-hydroxyindoles with 3,4-dimethoxybenzylamine. Curves: 1=5-hydroxytryptophan; 2=5-hydroxyindole-3-acetic acid; 3=serotonin; 4=5-hydroxyindole-3-acetamide; 5=*N*-acetyl-5-hydroxytryptamine; 6=5-hydroxytryptophol.

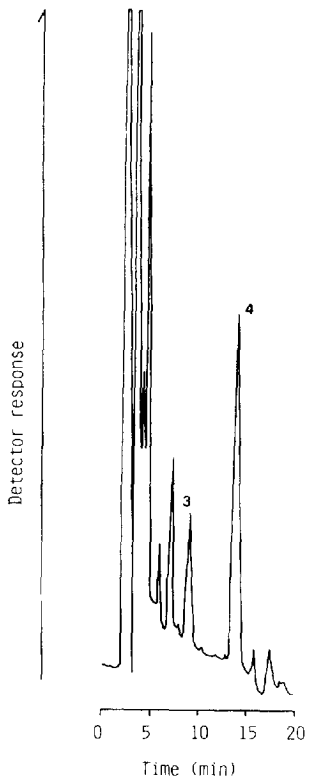

Fig. 3. Chromatogram obtained with human platelet-poor plasma from a healthy volunteer. Peaks: 3=serotonin; (14.8 pmol/ml plasma); 4=I.S..

3,4-dimethoxybenzylamine and/or potassium hexacyanoferrate(III), peaks 3 and 4 were not observed in the chromatogram even at high detector sensitivity. These results suggest that peaks 3 and 4 are fluorescent derivatives of serotonin and 5-hydroxyindole-3-acetamide, respectively. The peaks for *N*-acetyl-5-hydroxytryptamine and 5-hydroxytryptophol were not observed in the chromatogram from platelet poor plasma. On the contrary, 5-hydroxytryptophan and 5-hydroxyindole-3-acetic acid could not be successfully detected because some large peak(s) due to plasma endogenous substances other than the indoles appeared at retention times of 2–5 min on the chromatogram (Fig. 3). Studies on an improved method for the determination of these indoles are in progress.

The calibration graph of serotonin for the platelet poor plasma was linear (r=0.999) in the concentration range corresponding to 1–500 pmol/ml plasma. No significant change in the slope of the graph was observed with plasma used.

The lower limit of detection for serotonin was 450 fmol/ml platelet poor plasma (3 fmol/injection) at a signal-to-noise ratio of three. This sensitivity was approx. 7–80-times higher than that of HPLC with electrochemical detection [9–12].

The recoveries of serotonin and 5-hydroxyindole-3-acetamide (20 pmol/ml plasma each) added to pooled platelet-poor plasma were 64.9±2.0 and 74.3±2.3% (mean±S.D.; n=5). The within-day precision was established by repeated determination (n=10) of serotonin concentrations in platelet poor plasma (14.8 and 50.5 pmol/ml plasma); the relative standard deviation was 4.0 and 3.8%, respectively.

The concentration of serotonin in platelet poor plasma from healthy subjects (n=15), measured by the proposed method, was 15.9±9.1 pmol/ml. This value is similar to those obtained by other methods [9–14].

The study has provided the first HPLC method with selective fluorescence derivatization for the determination of serotonin in human platelet-poor plasma. The method is sensitive enough to measure serotonin in 40 μl of plasma. It is readily performed and could therefore be useful for the evaluation of serotonergic functions for diagnostic purposes and biological investigations where only a small amount of plasma is available.

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