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Journal of Chromatography B, 738 (2000) 199–206

JOURNAL OF CHROMATOGRAPHY B

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4-Dimethylaminobenzylamine as a sensitive chemiluminescence derivatization reagent for 5-hydroxyindoles and its application to their quantification in human platelet-poor plasma

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Received 27 July 1999; received in revised form 20 October 1999; accepted 27 October 1999

Abstract

A selective and sensitive high-performance liquid chromatographic method with chemiluminescence detection for the determination of 5-hydroxyindoles is described, based on the reaction of 5-hydroxyindoles with 4-dimethylaminobenzylamine. Serotonin, 5-hydroxyindole-3-acetic acid, 5-hydroxytryptophol, 5-hydroxyindole-3-acetamide and *N*-acetyl-5-hydroxytryptamine were used as model compounds to optimize the derivatization and chemiluminescent reaction. The reagent reacts with 5-hydroxyindoles in slightly alkaline media in the presence of potassium hexacyanoferrate(III) to give the corresponding derivatives, which can be separated on a reversed-phase column, Wakosil-II 5C18RS, with aqueous acetonitrile as an eluent. The derivatives were detected by peroxyoxalate chemiluminescence detection. The detection limits are in the range of 0.5–1.2 fmol per 100-μl injection. The method was applied to the simultaneous determination of serotonin and 5-hydroxyindole-3-acetic acid in human platelet-poor plasma.

Keywords: Derivatization, LC; 4-Dimethylaminobenzylamine; 5-Hydroxyindoles

1. Introduction

5-Hydroxyindoles are metabolites of tryptophan and play physiologically important roles in the human body. The 5-hydroxyindoles including serotonin, one of the active metabolites of tryptophan, and its metabolites, occur in the central nervous system and blood platelets. The determination of these 5-hydroxyindoles in biological samples is useful for the elucidation of tryptophan metabolism and for the diagnosis of the intestinal carcinoid [1,2] tumors and the mental disorders such as schizophrenia and migraine [3,4].

Various high-performance liquid chromatographic (HPLC) methods have been developed for the determination of 5-hydroxyindoles with fluorimetric [5–8] and electrochemical detection [9–11] based on the native fluorescence and oxidation of the substances, respectively. However, these detection methods are not always specific and sensitive for 5-hydroxyindoles.

In previous works, we reported that aromatic methylamines such as benzylamine and 3,4-dimethoxybenzylamine react selectively and sensitive-

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0378-4347/00/$ – see front matter
PII: S0378-4347(99)00503-4

ly with 5-hydroxyindoles in weakly alkaline media in the presence of potassium hexacyanoferrate(III) to give highly fluorescent oxazole derivatives [12]. Further, some fluorimetric HPLC methods using these reagents have been successfully applied to the determination of 5-hydroxyindoles in biological samples [13–15].

Recently, a detection system based on the peroxyoxalate chemiluminescence (CL) has been successfully used in HPLC analysis owing to the high sensitivity and selectivity [16–20]. The present study explores the use of further highly sensitive fluorogenic reagents for the CL detection of 5-hydroxyindoles; eleven different species of aromatic methylamine have been screened as pre-column derivatization reagents for 5-hydroxyindoles in HPLC with peroxyoxalate CL detection. The derivatization and CL reaction conditions have been studied and optimized for the HPLC analysis of 5-hydroxyindoles, using serotonin, 5-hydroxyindole-3-acetic acid (5HIAA), 5-hydroxytryptophol (5HOL), 5-hydroxyindole-3-acetamide (5HA) and *N*-acetyl-5-hydroxytryptamine (N-AcSer) as model systems. Of eleven aromatic methylamines tested, 4-dimethylaminobenzylamine (4-DMBA) was found to be the most sensitive as a peroxyoxalate CL derivatization reagent for 5-hydroxyindoles. Hence, the reagent was successfully applied to the simultaneous determination of serotonin and 5HIAA in human platelet-poor plasma. 5HA was used as an internal standard (I.S.).

2. Experimental

2.1. Chemicals and solutions

Deionized and distilled water, purified with a Milli-Q II (Millipore, Milford, MA, USA) system, was used for all aqueous solutions. Serotonin, 5HIAA, 5HOL, 5HA and *N*-AcSer were purchased from Sigma (St. Louis, MO, USA). 4-Dimethylaminobenzylamine hydrochloride (4-DMBA) was used after purification by recrystallization with ethanol. Other chemicals were of the highest purity available and were used as received. The 10 m*M* solutions of 4-DMBA and the other aromatic methylamines were prepared by dissolving the reagents in a mixture of dimethylsulfoxide (DMSO) and water (1:1, v/v); the regent solutions were stable for at least 1 week, even at room temperature. Potassium hexacyanoferrate(III) (30 m*M*) and sodium hydroxide (70 m*M*) solutions were prepared in a mixture of DM50 and water (1:1, v/v); both the solutions were stable for at least 1 day. Standard solutions of 5-hydroxyindoles were prepared in water and kept frozen (−20°C) in amber colored test-tubes. The 5 m*M* solutions of bis (2,4,6-trichlorophenyl)oxalate (TCPO), bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl]oxalate (TDPO) and bis[2-(3,6-dioxaoctyloxycarbonyl)phenyl]oxalate (DOPO) (all from Wako Pure Chemicals, Tokyo, Japan) were prepared by dissolving them in ethyl acetate. Imidazole was recrystallized twice from toluene and 120 m*M* imidazole buffer was prepared in water; the pH was adjusted with nitric acid. Hydrogen peroxide in aqueous solution (31%, w/w) (Mitsubishi Gas Kagaku, Tokyo, Japan) was diluted with a mixture of ethyl acetate–acetonitrile (1:9, v/v) to appropriate concentrations.

2.2. Chromatography

A schematic flow diagram of the HPLC system is illustrated in Fig. 1. A Hitachi L-7000 high-performance liquid chromatograph (Tokyo, Japan) equipped with a Rheodyne 7125 syringe-loading sample injector valve (100-μl loop) was used. 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), with isocratic elution with a mixture of acetonitrile and 50 m*M* Tris–HCl buffer (pH 6.0) (2:3, v/v) at a flow-rate of 0.8 ml/min. The column temperature was ambient (20–23°C).

In the post-column CL reaction system, 5 m*M* TCPO and 0.3 *M* hydrogen peroxide solutions were delivered at 0.5 ml/min each by a Jasco PU980 pump (Tokyo, Japan). Both the solutions were combined and mixed by passing through a stainless-steel mixing coil (5 m×0.5 mm I.D.). The eluate from the HPLC column was mixed with the CL reagent by the T-type mixing device. The CL generated in the eluate was monitored by a Jasco 825-CL intelligent CL detector equipped with a 90-μl flow-cell. Stainless-steel tubing (0.5 mm I.D.) was

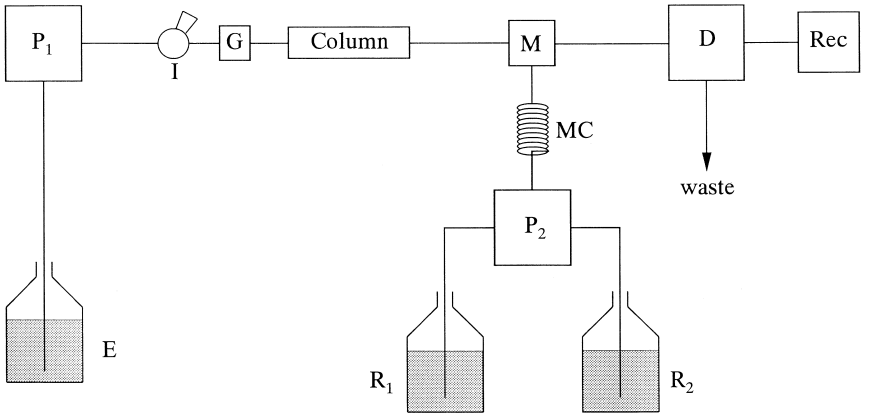

Fig. 1. Schematic flow diagram of the HPLC system with CL detection. P_1, HPLC pump for mobile phase; SI, sample injector valve; P_2, reagent delivery pump; G, guard column; M, mixing device; MC, mixing coil; D, CL detector; E, mobile phase; R_1, TCPO solution; R_2, hydrogen peroxide solution; Rec, recorder.

used for the HPLC–CL system. The CL was recorded on a Hitachi D-2500 Chromato-Integrator.

2.3. Fluorescence derivatization

To a 50-μl portion of a test solution of 5-hydroxyindoles were added 50 μl of 70 m*M* sodium hydroxide solution, 100 μl of 10 m*M* 4-DMBA (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 ca. 2 min. A 100-μl portion of the final reaction mixture was injected into the chromatograph. To prepare the reagent blank, 50 μl of water in place of 50 μl of a test solution was carried out through the procedure.

2.4. Human platelet-poor plasma sample

Platelet-poor plasma samples were prepared in the usual manner [21,22]. 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 of Na_2EDTA per 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 and 5-hydroxyindole-3-acetic acid 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 80 n*M* 5HA (I.S.) and 140-μl of 100 m*M* Tris hydrochloric acid buffer (pH 9.0) were pipetted into an 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 (50 μl) from the cartridge was treated according to the fluorescence derivatization.

For the establishment of the calibration graph, 20 μl of the 5HA (I.S.) solution was replaced by an I.S. solution containing serotonin and 5HIAA (10 fmol–100 pmol each per 20 μl). The net peak height ratios of serotonin (or 5HIAA) and I.S. were plotted against the concentrations of the 5-hydroxyindoles added.

3. Results and discussion

3.1. Selection of 4-dimethylaminobenzylamine as a pre-column derivatization reagent for peroxyoxalate CL detection

In a previous work [15], various aromatic methylamines were screened as pre-column derivatization

Table 1
Relative CL intensities (RCLI) and their retention times (t_R) of the derivatives produced by the reaction of 5HIAA with aromatic methylamines

Aromatic methylamine	t_R (min)	RCLI[a]
4-Dimethylaminobenzylamine	14.4	100.0
3,4-Dimethoxybenzylamine	6.4	12.8
2,3-Dimethoxybenzylamine	10.2	10.3
3,5-Dimethoxybenzylamine	12.4	11.7
Benzylamine	9.9	10.6
4-Methoxybenzylamine	10.2	11.7
4-Methylbenzylamine	15.6	10.8
3-Methylbenzylamine	16.0	8.7
4-Hydroxy-3-methoxybenzylamine	4.3	1.5
2-Aminomethylpyridine	3.4	16.0
3-Aminomethylpyridine	3.3	7.1

[a] The CL intensities (peak area) obtained by the reaction of 5HIAA with 4-DMBA was taken as 100.0.

reagents for 5-hydroyindoles by a HPLC method with fluorescence detection. In this work, eleven aromatic methylamines shown in Table 1 were studied as pre-column derivatization reagents in HPLC with peroxyoxalate CL detection using 5HIAA as a representative 5-hydroxyindole of biological importance. The CL intensities (peak area) and retention times of the derivatives are shown in Table 1. 4-DMBA provided the most intense CL; 4-DMBA hydrochloride was tentatively selected.

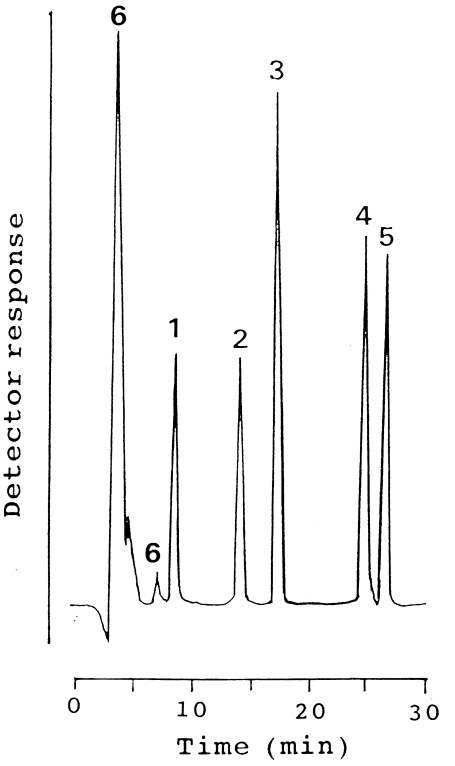

Fig. 2. Chromatogram of the derivatives of 5-hydroxyindoles. Peaks (0.33 pmol each on column): 1=serotonin; 2=5HIAA; 3=5HA; 4=*N*-AcSer (I.S.); 5=5HOL; 6=reagent blank.

3.2. HPLC condition

The best separation of serotonin, 5HIAA, 5HA, *N*-AcSer and 5HOL was achieved on a reversed-phase column, Wakosil-II 5C18RS, with a mixture of acetonitrile and 50 m*M* Tris–HCl buffer (pH 6.0) (2:3, v/v) as mobile phase. Five 5-hydroxyindoles could be separated by isocratic elution within 30 min under the HPLC conditions. Fig. 2 shows a typical chromatogram obtained with a standard mixture of the 5-hydroxyindoles tested; their retention times (min) were 8.6 (5HT), 14.4 (5HIAA), 17.7 (5HA), 25.5 (*N*-AcSer) and 27.3 (5HOL).

3.3. Derivatization conditions

In the present work, we found that the optimal fluorescence derivatization conditions of 5-hydroxyindoles with 4-DMBA were slightly different from those with benzylamine and 3,4-dimethoxybenzylamine [12–15]. The optimal conditions for 4-DMBA were examined using the five 5-hydroxyindoles. DMSO allowed the most intense peaks at concentrations between 35 and 50% (v/v) in the reaction mixture; 40% was selected tentatively. The potassium hexacyanoferrate(III) solution gave the most intense peaks at a concentration greater than 20 m*M*; 30 m*M* was used as a sufficient concentration. The 4-DMBA solution gave the most intense peaks at concentrations of 10 m*M* and 10–20 m*M* for *N*-AcSer and the other indoles, respectively; 10 m*M* was adopted for the recommended procedure. Maximum peaks were obtained with sodium hydroxide at concentrations between 60 and 75 m*M* in the solution; 70 m*M* was employed for the present procedure.

The derivatization reaction of 4-DMBA with 5-hydroxyindoles occurred very rapidly even at 0°C.

The peak heights for the five 5-hydroxyindoles reached maximum and constant values after standing at room temperature for >1 min. Therefore, standing at room temperature for 2 min was selected in the recommended procedure.

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

3.4. CL reaction in HPLC

Various parameters such as the pH and concentration of imidazole buffer, the kind and concentration of oxalate, the hydrogen peroxide concentration are well known to affect the peroxyoxalate CL response [16–20]. In this study, the optimum conditions were examined for the 4-DMBA derivatives of 5-hydroxyindoles.

Imidazole was necessary for the development of the peroxyoxalate CL reaction with the 4-DMBA derivatives of 5-hydroxyindoles. In the HPLC system, the optimum pH of the imidazole buffer was pH 6.0 (Fig. 3A). The imidazole buffer (pH 6.0) gave the most intense peaks at concentrations between 10 and 100 m*M*. Imidazole–HCl buffer (50 m*M*, pH 6.0) was selected because this gave the highest signal-to-noise ratios for the 5-hydroxyindole peaks. The TCPO solution gave the most intense peaks at concentrations of 4–10 m*M* Fig. 4A); 5 m*M* was adopted for the recommended procedure. On the other hand, the peak heights obtained with TDPO and DOPD gave about 70 and 20%, respectively, of those with TCPO. Maximum peaks were obtained with hydrogen peroxide at concentrations between 0.4 and 0.7 *M* in the solution (Fig. 4B); 0.5 *M* was employed for the present procedure.

The optimum CL conditions for the 4-DMBA derivatives of 5-hydroxyindoles were almost the same as those for some analytes such as dansyl-alanine, reported in previous papers [16–20].

3.5. Validation of the method

The relationships between the peak heights and the amounts of the individual 5-hydroxyindoles were linear up to at least 100 pmol per injection volume (100 μl).

In order to confirm the detection limits, a standard mixture of the five indoles (3 fmol each on column) was treated as in the procedure (Fig. 5). The figure indicates that the detection limits (fmol, signal-to-noise ratio=3) were 1.2 (serotonin), 1.2 (5HIAA), 0.5 (5HA), 0.7 (*N*-AcSer) and 0.7 (5HOL) for an injection volume of 100 μl; the sensitivity is ca. five to ten times higher than the fluorescence detection with benzylamine and 3,4-dimethoxybenzylamine. The peaks due to the reagent blank components (peak 6 in Fig. 5A and B) did not disturb the highly

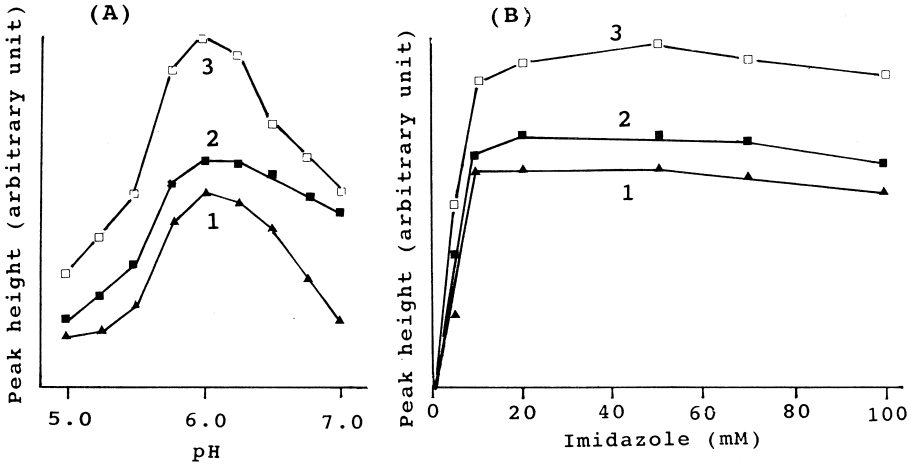

Fig. 3. Effects of (A) pH and (B) concentration of the imidazole buffer on CL peak heights for the 4-DMBA derivatives of 5-hydroxyindoles. Curves: 1=serotonin; 2=5HOL; 3=5HA.

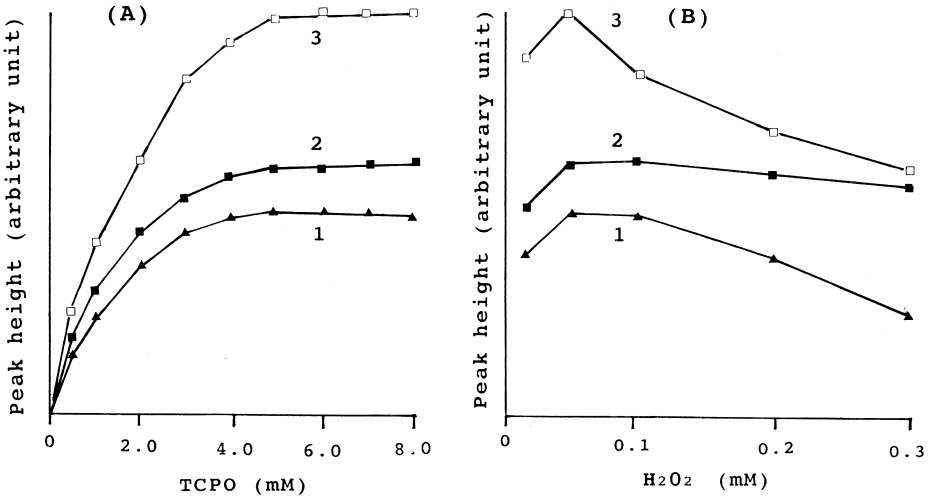

Fig. 4. Effects of the concentrations of (A) TCPO and (B) hydrogen peroxide on the CL peak heights for the 4-DMBA derivatives. Curves: 1=serotonin; 2=5HOL; 3=5HA.

sensitive determination of the indoles even at extremely low concentrations.

The within-day reproducibility of the CL detection was established by repeated determination of a standard mixture of the 5-hydroxyindoles (1.0 nmol each per ml). The relative standard deviations did not exceed 5.9% for all the indoles examined ($n=10$ in each case).

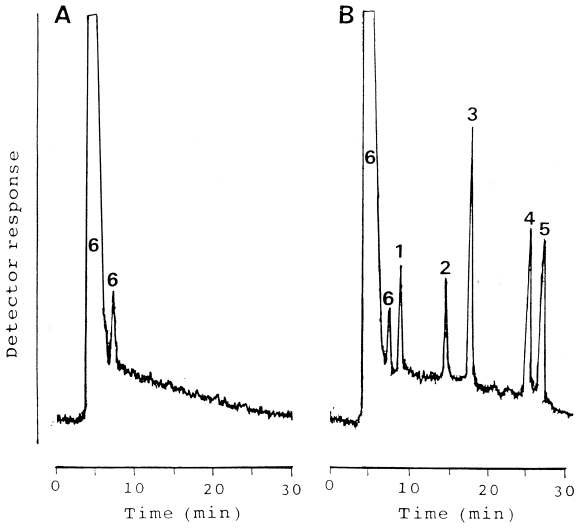

Fig. 5. Chromatograms obtained with (A) a blank sample and (B) a standard mixture of 5-hydroxyindoles at low concentrations. Peaks (3 fmol each on column): see the legend of Fig. 2.

3.6. Determination of serotonin and 5-hydroxyindole-3-acetic acid in human platelet-poor plasma

Plasma was treated as in the procedure described previously [15].

Typical chromatograms obtained with platelet-poor plasma from a healthy subject and a carcinoid patient are shown in Fig. 6A and B, respectively. Peaks 1, 2 and 4 correspond to serotonin, 5HIAA 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 20–50% (v/v) acetonitrile as mobile phase. When the analysis was performed without 4-DMBA and/or potassium hexacyanoferrate(III), peaks 1, 2 and 4 were not observed in the chromatogram even at high detector sensitivity. These results suggest that peaks 1, 2 and 4 are fluorescent derivatives of serotonin, 5HIAA and 5HA, respectively. The peaks for *N*-AcSer and 5HOL were not observed in the chromatograms from platelet-poor plasma.

The calibration graphs of serotonin and 5HIAA for the platelet-poor plasma were linear ($r=0.998$ for

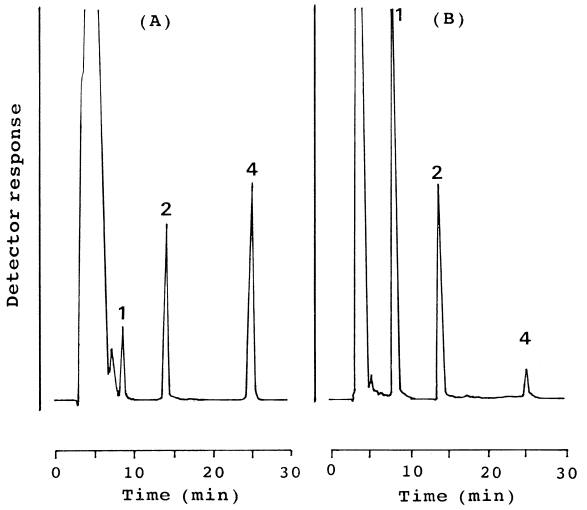

Fig. 6. Chromatograms obtained with platelet-poor plasma samples from (A) a healthy volunteer and (B) a carcinoid patient. Peaks: 1=serotonin [(A) 20.2 pmol and (B) 1510.2 pmol/ml plasma]; 2=5HIAA [(A) 50.5 pmol and (B) 490.1 pmol/ml plasma]; 4=*N*-AcSer (I.S.). Detector sensitivity: (A)×8; (B)×1.

both the indoles) in the concentration range corresponding to 1–2000 pmol/ml plasma. No significant changes in the slopes of the graphs were observed with plasma used.

The lower limits of detection for serotonin and 5HIAA were 375 and 250 fmol/ml, respectively for platelet-poor plasma (1.5 and 1.0 fmol, respectively per injection) at a signal-to-noise ratio of three. This sensitivity is ca. 10–100 and three to five times higher than those of HPLC with electrochemical [21–24] and fluorescence detection [15], respectively.

The recoveries of serotonin, 5HIAA and 5HA (20 pmol each per ml plasma each) added to pooled platelet-poor plasma were 77.3±4.2, 72.2±3.7 and 75.4±4.3% (mean±SD; $n=10$), respectively. The within-day precision was established by repeated determination ($n=10$) of serotonin and 5HIAA concentrations in platelet-poor plasma (17.7 and 20.5 pmol each per ml plasma); the relative standard deviations were 5.0 and 4.8%, respectively. The concentrations (mean±SD) of serotonin and 5HIAA measured by the proposed method were respectively as follows: 16.8±10.1 and 50.5±9.8 pmol/ml in platelet-poor plasma from healthy subjects ($n=10$); 1560.2±1838.2 and 505.2±215.3 pmol/ml in platelet-poor plasma from carcinoid patients ($n=4$). These values are similar to those obtained by other methods [14,15,21–26].

The study has provided the first HPLC method with selective fluorescence derivatization and peroxyoxalate CL detection for the simultaneous determination of serotonin and 5HIAA in human platelet-poor plasma. The method is sensitive enough to measure serotonin and 5HIAA in 40 μl of plasma. The method is readily performed and could therefore be useful for evaluation of serotonergic functions for diagnostic purposes and biological investigations where only a small amount of plasma is available.

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