Selective and sensitive liquid chromatographic determination method of 5-hydroxyindoles with fluorous and fluorogenic derivatization

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Abstract

A liquid chromatographic (LC) method with improved selectivity for the simultaneous determination of 5-hydroxyindoles (5-HIs; 5-hydroxytryptophan, 5-hydroxytryptamine, *N*-acetyl-5-hydroxytryptamine, 5-hydroxyindole-3-acetic acid, and 5-hydroxytryptophol) is described. This method involves precolumn derivatization with 4-(3',3',4',4',5',5',6',6',7',7',8',8',9',9',10',10',10'-heptadecafluorodecyl)benzylamine (HFBA) and separation of the derivatives using a fluorous LC column. In this study, stable benzoxazole derivatives of 5-HIs with HFBA has been obtained by a simple derivatization procedure; their fluorescent properties enabled highly sensitive detection. In addition, only the HFBA derivatives of 5-HIs has been selectively retained on the fluorous LC column via fluorous interaction whereby perfluoroalkyl compounds show affinities with each other, while the non-fluorous compounds did not. The HFBA derivatives were separated within 30 min and the detection limits for 5-HIs in a 20- μ L injection volume are 1.2–14 fmol ($S/N = 3$). Furthermore, this method was applied to the analysis of 5-HIs in the human plasma from healthy subjects.

33 **Keywords**

34 5-Hydroxyindoles, Fluorous derivatization, Liquid chromatographic-fluorescent
35 detection, Fluorous separation, Human plasma

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38 **1. Introduction**

39 5-Hydroxyindoles (5-HIs), originating from tryptophan, are produced via the
40 corresponding biosynthetic pathways in the human body [1] and play important roles in
41 the control and regulation of the functions of the central and peripheral nervous
42 systems; they also exhibit associations with various diseases [2–5]. In particular, 5-
43 hydroxytryptamine (5-HT) occurs mainly in the central nervous system, blood platelets,
44 and enterochromaffin cells of the gastrointestinal tract. 5-HT is sequentially
45 metabolized, first by monoamine oxidase and then by aldehyde dehydrogenase, to form
46 5-hydroxyindole-3-acetic acid (5-HIAA). 5-HIAA is the most abundant end product of
47 both central and peripheral 5-HT metabolism and is excreted into urine. The
48 correlations of 5-HIAA levels in plasma and urine to some pathological conditions have

been reported [4,5]. Thus, a simple, selective, sensitive, and simultaneous determination method of 5-HIs in the human body is of great importance in the research involving 5-HIs or the diagnosis of diseases related to 5-HIs.

A number of methods have been described for the determination of 5-HIs; of these, liquid chromatography (LC) with native fluorescent detection [6–8], electrochemical detection [9–11], and mass spectrometry (MS) [12–16] have been most widely used. Although these methods are reliable, a number of peaks attributed to coinciding compounds in biological samples may interfere with the corresponding chromatogram expect for LC-MS. Therefore, highly sophisticated LC separation conditions and troublesome clean-up procedures are required to eliminate the interferences caused by other coexisting compounds. In contrast, MS and MS/MS detection methods are selective, sensitive, and highly reliable; however, these techniques are sometimes interfered with by matrix components present in biological samples and accurate determination may not be obtained. [17,18].

We have previously reported selective and sensitive fluorogenic derivatization methods for 5-HIs and catecholamines in biological samples [19–24]. These methods

were based on the reactions of these analytes (weakly fluorescent) with non-fluorescent benzylamine (BA) in a weakly alkaline medium in the presence of an oxidizing agent, yielding highly fluorescent benzoxazole derivatives. Furthermore, we have also applied some BA derivatives as fluorogenic derivatization reagents for 5-HIs and catecholamines, resulting in improved analytical sensitivities for their detection [25–28].

Recently, we have also developed LC analytical methods for native fluorescent compounds based on “fluorous derivatization,” in which “fluorous” refers to the unique affinity between highly fluorinated compounds [29–31]. The principle of this method is that native fluorescent analytes are derivatized precolumn by attachment of a non-fluorescent fluorous compound to a particular functional group; thereafter, the fluorous-derivatized analytes are extremely retained on a fluorous phase LC column, thus achieving selective separation of analytes from biological sample matrices. Neither non-fluorescent compounds nor underivatized native fluorescent compounds interfere with the detection. This method was successfully applied to the determination of pharmaceutical agents possessing carboxylic groups in the human plasma and urine

after administration [30], as well as native fluorescent biogenic amines in the human urine [31].

In this study, we report a novel derivatization method for the highly selective and sensitive determination of 5-HIs by combining both of the above-mentioned fluororous and fluorogenic derivatization methods. To the best of our knowledge, this is the first report on analyses with both fluorogenic derivatization using a fluororous labeling reaction. 5-Hydroxytryptophan (5-HTRP), 5-HT, *N*-acetyl-5-hydroxytryptamine (NAS), 5-HIAA, and 5-hydroxytryptophol (5-HTOL) were selected as model 5-HI compounds (Fig. 1). 5-HTRP, NAS, and 5-HTOL as well as 5-HT and 5-HIAA are produced from tryptophan in human body and compose the biosynthetic and metabolic pathways of serotonin [1]. 4-(3',3',4',4',5',5',6',6',7',7',8',8',9',9',10',10',10'-Heptadecafluorodecyl)benzylamine (HFBA), possessing a fluororous moiety and benzylamine structure, was used as a derivatization reagent (Fig. 1). This determination method has both high selectivity based on fluororous interactions and high sensitivity based on fluorogenic derivatization. The conditions for derivatization, LC separation, and fluorescent detection were optimized using standard solutions of 5-HIs. After

optimization studies, this method was applied to the analysis of 5-HT and 5-HIAA in the human plasma from healthy subjects without the need for any other enrichment or clean-up procedure.

Fig. 1

2. Experimental

2.1. Reagents and materials

Unless stated otherwise, all chemicals mentioned below were of the highest purity available and were used as received. 5-HTRP (5-hydroxytryptophan dihydrate), 5-HTOL, and HFBA were purchased from Sigma–Aldrich (St. Louis, MO, USA). 5-HT (5-hydroxytryptamine hydrochloride), 5-HIAA, and benzylamine hydrochloride (BA) were purchased from Wako Pure Chemicals (Osaka, Japan). NAS, *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS), and potassium hexacyanoferrate(III) were purchased from Tokyo Chemical Industry (Tokyo, Japan), Dojindo Laboratories (Kumamoto, Japan), and Kishida Chemical (Osaka, Japan), respectively. All organic solvents were of LC grade. It should be noted that these reagents and solvents are toxic

to the eyes, lungs, and skin, and should be used carefully according to guidelines specified in the latest material safety data sheets. Ultrapure water, purified using a Milli-Q gradient system (Merck Millipore, Billerica, MA, USA), was used to produce all aqueous solutions.

The respective stock solutions (1.0 mM) of analytes were prepared in water and stored at $-30\text{ }^{\circ}\text{C}$. The solutions were stable for at least 1 week and diluted further with ultrapure water to the required concentrations before use. A solution of 10-mM HFBA in tetrahydrofuran (THF) was usable for at least 1 week when stored at room temperature. A CAPS buffer (30 mM) was prepared in water and adjusted to pH 12 with aqueous sodium hydroxide. Potassium hexacyanoferrate(III) solution (20 mM) was prepared using the above-prepared 30-mM CAPS buffer (pH 12) and used within 1 d.

2.2. *Derivatization procedure*

To the sample solution (200 μL) placed in a polypropylene 1.5-mL vial were added 125 μL of 10-mM HFBA and 25 μL of 20-mM potassium hexacyanoferrate(III). The vial was sealed, and the mixture was left at room temperature for 2 min. Thereafter,

350 μ L of aqueous 60% (v/v) acetonitrile was added to the mixture, and the entire reaction solution was placed in the autosampler of an LC system. A reagent blank was prepared by the same procedure using a 200- μ L aliquot of aqueous 75% (v/v) THF instead of the sample solution.

2.3. *Plasma sample and pretreatment*

Plasma samples were obtained from the whole blood of healthy volunteers in our laboratory. These volunteers understood the purpose and the significance of this experiment and donated blood after signing an agreement. The whole-blood samples were collected from five volunteers (three males aged from 23 to 25 and two females aged 24) using a centrifugal blood-collection tube (Eiken Chemical, Tokyo, Japan). The blood was immediately centrifuged at 2,000 $\times g$ for 5 min at room temperature, and the obtained supernatant was transferred to a screw-capped polypropylene tube and used as a plasma sample.

To 70 μ L of plasma placed in a 0.5-mL polypropylene tube was added 210 μ L of THF. After vortex mixing for a few seconds, the mixture was immediately centrifuged

at $9,000 \times g$ for 5 min at 4 °C, and the supernatant was passed through a disposable filter (0.20- μ m pore size, polytetrafluoroethylene; Advantec Toyo, Tokyo, Japan). Then, a portion (200 μ L) of the filtrate was subjected to derivatization.

2.4. Instrumentation and conditions

The analyses of fluororous–fluorescence derivatized 5-HIs by LC were performed using a Waters AllianceTM 2695 system (Waters, Milford, MA, USA) consisting of a binary pump, an on-line degasser, an autosampler, and a column oven connected to a fluororous-phase Fluofix-II 120E column (150 $\times$ 4.6 mm i.d., particle size 5 μ m; Wako Pure Chemicals) and Waters 2475 fluorescence detector equipped with an 8- μ L flow cell. The injection volume was 20 μ L. Chromatographic control, data acquisition, and analyses were performed using Empower 2 software (Waters). The reacted solutions in sample vials were stored at room temperature in the autosampler contained in the Waters AllianceTM 2695 system. The fluorescence detector was operated at an excitation wavelength of 340 nm and emission wavelength of 452 nm. A mixture of methanol–water–acetonitrile–trifluoroacetic acid (80:13:7:0.1 v/v) was used as a mobile

phase for isocratic elution. The flow rate was set at 1.0 mL/min and the column was maintained at ambient temperature.

2.5. *Fluorescence spectral characterization*

Fluorescence spectral measurements were performed using a Waters 2475 fluorescence detector. Other conditions, except for the detection conditions, were the same as those described in the previous section (Section 2.4). On-line fluorescence spectra were obtained using peak components of the HFBA-derivatized 5-HT and 5-HTRP on LC. The excitation spectra were monitored at 280–380 nm while operating the detector with an emission wavelength of 452 nm; the emission spectra were monitored at 375–560 nm with excitation at 340 nm. The slit widths of both the monochromators were set at 20 nm.

2.6. *Comparison of fluoruous and non-fluoruous derivatives*

In order to examine the selectivity of fluoruous separation, the derivatization of 5-HIs with the non-fluoruous reagent BA (which has no fluoruous structure) was carried out

using the same procedure as outlined above (Section 2.2), expect for the type of derivatization reagent used. The non-fluorous derivatives were analyzed using the same LC conditions.

2.7. Validation study

The concentrations of 5-HIs were calculated from the calibration curves by using the peak areas of respective 5-HIs. For the quantitative analysis, standard solutions for calibration with concentrations ranging from 0.04–2 μM (0.04, 0.1, 0.2, 1, and 2 μM ; 5-HT, 5-HIAA, NAS, and 5-HTOL) and 0.2–10 μM (0.2, 0.5, 1, 5, and 10 μM ; 5-HTRP) were prepared by diluting the standard solutions. For the plasma sample, spiked solutions for calibration with concentrations ranging from 0 to 300 pmol/mL plasma (0, 15, 30, 75, and 300 pmol/mL plasma; 5-HT, 5-HIAA, NAS, and 5-HTOL) and 0 to 1500 pmol/mL plasma (0, 75, 150, 375, and 1500 pmol/mL plasma; 5-HTRP) were prepared by spiking the human plasma ($n = 3$). The equations of the calibration curves were determined using least squares linear prediction.

The precision (intraday and interday) of peak area in the present method was estimated during the analytical procedures (pretreatment, derivatization, LC separation, and detection) using the standard solutions (5-HTRP, 0.2 and 10 μ M; other 5-HIs, 0.04 and 2 μ M) and spiked plasma (5-HTRP, 150 pmol/mL plasma; other 5-HIs, 30 pmol/mL plasma). The intraday and interday precision values were assessed by performing the analysis six times on the same day and by conducting the analysis on six different days within a month, respectively.

To demonstrate applicability to real samples, the recoveries using plasma samples spiked with standards were evaluated. For this purpose, a standard solution was added to the plasma sample (5-HTRP, 150 pmol/mL plasma; other 5-HIs, 30 pmol/mL plasma), and the recovery was calculated on the basis of the results obtained by the proposed method. The recovery (R) was calculated as

$$R = 100 \times \{(C_{\text{spike}} - C_{\text{sample}})/C_{\text{standard}}\}, \quad (1)$$

where C_{spike} is the concentration in the spiked plasma sample, C_{sample} is the concentration in the plasma sample without spiking, and C_{standard} is the added concentration.

207 The limit of detection (LOD) was determined as the lowest amounts on column
208 and concentrations in standard solution for which the signal-to-noise ratio was 3. The
209 lower limit of quantification (LLOQ) was determined as the lowest concentration in
210 plasma for which the signal-to-noise ratio was 5.

211

212 **3. Results and discussion**

213 *3.1. Fluorescent properties of derivatives*

214 To optimize the fluorescence detection conditions for the determination of HFBA-
215 derivatized 5-HIs, on-line fluorescence spectra were measured and compared with the
216 BA-derivatized 5-HIs. The fluorescence excitation and emission maxima obtained with
217 HFBA-derivatized 5-HIs and BA-derivatized 5-HIs are shown in Table 1. As expected,
218 the HFBA-derivatized 5-HIs exhibited the fluorescence with longer wavelength, similar
219 to BA-derivatized 5-HIs, than the underivatized 5-HIs themselves (λ_{ex} 280 nm, λ_{em} 340
220 nm) [29]. However, the maximum emission wavelengths obtained with HFBA-
221 derivatized 5-HIs were shifted to slightly shorter wavelengths around 450 nm compared
222 to BA-derivatized 5-HIs (465 nm). Therefore, 340 nm and 452 nm were chosen as the

optimal excitation and emission wavelengths for the determination of 5-HIs. These data suggest that the HFBA-derivatized 5-HIs have fluorescent property as well as BA-derivatized 5-HIs [19–24].

Table 1

3.2. Fluorous LC separation

Typical chromatograms of the derivatized 5-HIs are provided in Fig. 2. The HFBA-derivatized 5-HIs were strongly retained on the fluororous LC column (Fig. 2A). Following derivatization with BA, however, the non-fluorous derivatized 5-HIs were easily eluted under the same LC conditions (Fig. 2B). These chromatograms indicate that the HFBA-derivatized 5-HIs were selectively retained on the fluororous LC column owing to their fluorophilicity and not their hydrophobicity. In addition, fluororous LC columns have been demonstrated to separate compounds not only on the basis of fluorophilicity, but also by secondary separation effects derived from the significant number of silanol residues in silica gel-based fluorinated phases [32–36]. In this study, to further tune the analyte retention on the fluororous LC column and to allow the

separation of HFBA-derivatized 5-HIs, we examined different types of LC columns, namely, Fluofix-II 120E, Fluophase RP (Thermo Fisher Scientific, San Jose, CA, USA), and FluoroFlash (Fluorous Technologies, Pittsburgh, PA, USA), and the effects of mobile phase on the retention and separation of the derivatives. HFBA-derivatized 5-HIs could be retained and separated on all fluoruous columns examined by mainly fluorophilicity and partially hydrophobic and hydrophilic effects; the best separation of the HFBA-derivatized 5-HIs was achieved on the LC conditions as described in the Experimental section (Section 2.4). The retention and elution of derivatives on the fluoruous LC columns could be controlled by use of fluorophilic solvents for the elution from the column, such as acetonitrile and 2,2,2-trifluoroethanol, and hydrophilic and hydrophobic solvents for the mutual separation of the derivatives, such as water and methanol and/or their mixtures as a mobile phase.

Fig. 2

3.3. Derivatization conditions

Optimization studies for the derivatization were carried out to maximize the fluorescence peak area on LC using five 5-HI solutions (5-HTRP, 5 μ M; other 5-HIs, 1 μ M). Because HFBA did not easily dissolve in water, the derivatization reaction proceeded in the presence of organic solvents (methanol, acetonitrile, and THF). Best results were obtained when fluorophilic THF was used for the preparation of HFBA solution. By varying the concentration of HFBA over a concentration range 0.1–20 mM, the maximum peak areas of all derivatized 5-HIs were obtained by adding more than 5 mM of HFBA. Therefore, a 10-mM HFBA in THF was chosen and used in all further analyses. Next, the effect of potassium hexacyanoferrate(III) concentration (0.1–40 mM) was examined. The concentration of potassium hexacyanoferrate(III) that produced the maximum peak areas for all derivatized 5-HIs was more than 10 mM. Therefore, a 20-mM potassium hexacyanoferrate(III) was chosen as the optimum. Furthermore, the effect of the pH of the CAPS buffer (pH 8–12) was next examined. The maximum peak areas of all derivatized 5-HIs were obtained by adding the CAPS buffer (30 mM) with a pH of 12. Owing to the difficulty of preparing CAPS buffer with a pH higher than 12, the optimal pH was set to 12. Furthermore, the effects of reaction

time (0–60 min) at room temperature and 60 °C were examined. A reaction time over 1 min was required to obtain the largest peak with constant areas at room temperature and 60 °C. Therefore, the optimal derivatization reaction time and temperature for 5-HIs with HFBA was 2 min and room temperature, respectively.

Consequently, we chose 10-mM HFBA in THF, 20-mM potassium hexacyanoferrate(III) in 30-mM CAPS buffer at pH 12, and a reaction time of 2 min at room temperature as optimal for this study. Aqueous 60% (v/v) acetonitrile was added to terminate the derivatization reaction. The fluoruous-derivatized 5-HIs in the reaction mixture were stable for at least 1 d in dark at 4 °C.

3.4. Analysis of standards

Validation data for standard samples are presented in Table 2. The relationship between the amounts of 5-HIs examined and peak area were linear over the range 0.23–11 pmol (5-HT, 5-HIAA, NAS, and 5-HTOL) and 1.1–57 pmol (5-HTRP) on column. The determination coefficient values of 5-HIs were 0.9987–0.9996 ($n = 3$). The intraday and interday peak-area precisions were estimated during the entire process by repeated

determinations ($n = 6$ in each case) using two concentration standards. The relative standard deviations from intraday samples were within 4.1% and those from interday samples were within 8.7% (Table 2). The LOD (signal-to-noise ratio = 3) of 5-HIs were less than 14 fmol per 20- μ L injection volume, which corresponded to 2.5 pmol/mL in the sample solution (Table 2). The sensitivity of the present method was almost the same with the previous LC methods using BA as a fluorogenic derivatization reagent [21] or MS detection [15, 16]. Furthermore, that was superior to the native fluorescence detection [8] or electrochemical detection [11]. Thus, the present method allowed for highly sensitive determination of 5-HIs based on fluorogenic derivatization.

Table 2

3.5. Analysis of human plasma samples

The applicability of the present method to biological samples was confirmed by analyzing 5-HIs in the human plasma collected from five healthy volunteers. The human plasma was diluted 4-fold with THF to deproteinize the sample and to optimize the reaction solvent. Fig. 3 shows typical chromatograms obtained from a derivatized

plasma sample (A) with and (B) without spiking the standards. The components of peaks 1–5 in Fig. 3 were identified as the fluoruous-derivatized 5-HIAA, 5-HTOL, NAS, 5-HTRP, and 5-HT, respectively, by comparing both their retention times with those shown in Fig. 2 and cochromatography with standards using various mobile phases such as the mixtures of methanol–water–acetonitrile–trifluoroacetic acid (75~85:10~15:5~10:0.05~0.15 v/v). Non-fluoruous fluorescent compounds in plasma were not retained on the fluoruous LC column and eluted within 7 min. Therefore, no peaks due to plasma components without 5-HIs were observed at the retention times near to and beyond the objective peaks.

Validation data for the spiked plasma samples are presented in Table 3. The calibration graphs of 5-HIs in plasma were linear ($r^2 = 0.9978\text{--}0.9996$) in the concentration range corresponding to 0–300 pmol/mL plasma (5-HT, 5-HIAA, NAS, and 5-HTOL) and 0–1500 pmol/mL plasma (5-HTRP). The recoveries of 5-HIs from plasma samples spiked with standards were evaluated (5-HTRP, 150 pmol/mL plasma; other 5-HIs, 30 pmol/mL plasma). The examined 5-HIs were recovered from the human plasma in the range 68.2–104% (Table 3). Thus, the recoveries demonstrate that this

method allows for simultaneous determination of five 5-HIs without interference from contaminants in the human plasma. The intraday and interday peak-area precisions were established by repeated determinations ($n = 6$) of 5-HIs in the spiked plasma (5-HTRP, 150 pmol/mL plasma; other 5-HIs, 30 pmol/mL plasma); the relative standard deviations of intraday samples were within 8.4% and those of interday samples were within 8.0% (Table 3). The LLOQ (signal-to-noise ratio = 5) values were less than 17 pmol/mL plasma.

The concentrations of 5-HIs in the healthy human plasma samples were determined by the proposed method. The values (mean $\pm$ SD) of 5-HT and 5-HIAA were 16.7 ± 4.71 and 31.1 ± 14.9 pmol/mL plasma (Table 4), respectively, all of which are in good agreement with previously reported data [37,38].

Fig. 3, Table 3, and Table 4

4. Conclusion

This paper reports the development and application of the determination of 5-HIs using fluororous–fluorescent derivatization, fluororous-phase separation, and long-

wavelength fluorescent detection. This derivatization method exhibits both high selectivity based on fluorous interaction and high sensitivity based on fluorogenic derivatization. Therefore, this method could selectively and sensitively analyze 5-HIs with simple pretreatment and derivatization, and detect the requisite analytes without interference from contaminants in the human plasma samples. It is expected that this method could be applied as a more sensitive analytical technique for very diluted and small samples such as microdialysates by using a fluorous column-switching LC system [39] and/or fluorous solid-phase microextraction technique [40].

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Figure Captions

Figure 1

Fluorous–fluorogenic derivatization reaction for 5-HIs with HFBA.

Figure 2

(A) Typical chromatogram of standard 5-HIs derivatized with HFBA. Peaks and amounts (pmol on column): 1, 5-HIAA (5.7); 2, 5-HTOL (5.7); 3, NAS (5.7); 4, 5-HTRP (29); 5, 5-HT (5.7).

(B) Chromatogram of standard 5-HIs derivatized with BA under the same LC conditions as for (A).

Figure 3

(A) Chromatogram of 5-HIs derivatized with HFBA in spiked human plasma. Peaks and spiked concentration (pmol/mL plasma): 1, 5-HIAA (20); 2, 5-HTOL (20); 3, NAS (20); 4, 5-HTRP (100); 5, 5-HT (20).

(B) Chromatogram of 5-HIs derivatized with HFBA in normal human plasma. Peaks and concentrations (pmol/mL plasma): 1, 5-HIAA (10.8); 5, 5-HT (11.5).

Table 1 Fluorescence excitation and emission maxima of HFBA-derivatized 5-HIs and BA-derivatized 5-HIs.

Compound	HFBA derivative		BA derivative	
	λ_{ex} (nm)	λ_{em} (nm)	λ_{ex} (nm)	λ_{em} (nm)
5-HT	330	445	330	465
5-HIAA	330	445	330	465
NAS	335	455	335	465
5-HTOL	335	455	335	465
5-HTRP	330	430	330	465

Table 2 Validation data of standard 5-HIs.

Compound	r^2 ^a	LOD		RSD (% , $n = 6$)			
		fmol ^b	pmol/mL ^c	Intraday		Interday	
				High	Low	High	Low
				concentration ^d	concentration ^e	concentration ^d	concentration ^e
5-HT	0.9996	3.7	0.65	4.0	3.0	4.8	8.5
5-HIAA	0.9995	1.3	0.22	1.9	3.4	6.1	8.7
NAS	0.9991	1.2	0.21	2.2	4.1	3.3	5.7
5-HTOL	0.9987	1.2	0.21	1.6	3.8	6.4	5.0
5-HTRP	0.9989	14	2.5	4.0	3.9	6.3	5.3

^a Correlation curve in the range 0.23–11 pmol (5-HT, 5-HIAA, NAS, and 5-HTOL) and 1.1–57 pmol (5-HTRP) per 20-μL of injection volume.

^b Defined as the amount in 20-μL injection volume providing a signal-to-noise ratio of 3.

^c Defined as the concentration of sample solution affording a signal-to-noise ratio of 3.

^d Relative standard deviation obtained with peak area in 11 pmol (5-HT, 5-HIAA, NAS, and 5-HTOL) and 57 pmol (5-HTRP) per 20-μL injection volume.

^e Relative standard deviation obtained with peak area for 0.23 pmol (5-HT, 5-HIAA, NAS, and 5-HTOL) and 1.1 pmol (5-HTRP) per 20-μL injection volume.

Table 3 Validation data of 5-HIs in spiked human plasma.

Compound	r^2 ^a	LLOQ (pmol/mL) ^b	Recovery (%) ^c	RSD (% , $n = 6$) ^d	
				Intraday	Interday
5-HT	0.9996	4.3	84.2	5.6	6.7
5-HIAA	0.9989	1.5	72.2	5.6	4.8
NAS	0.9990	1.4	104	2.9	4.3
5-HTOL	0.9982	1.4	103	3.0	6.1
5-HTRP	0.9978	17	68.2	8.4	8.0

^a Correlation curve in the range 0–300 pmol/mL (5-HT, 5-HIAA, NAS, and 5-HTOL) and 0–1500 pmol/mL (5-HTRP) spiked plasma.

^b Defined as the concentration in plasma affording a signal-to-noise ratio of 5.

^c Recovery was calculated as follows: $\text{recovery} = 100 \times \frac{C_{\text{spike}} - C_{\text{sample}}}{C_{\text{standard}}}$, where C_{spike} is the concentration in the spiked plasma sample, C_{sample} is the concentration in the plasma sample without spiking, and C_{standard} is the concentration added ($n = 3$ each). Spiked concentrations (pmol/mL plasma): 5-HTRP (150), other 5-HIs (30).

^d Relative standard deviation obtained with peak area for 30 pmol/mL (5-HT, 5-HIAA, NAS, and 5-HTOL) and 150 pmol/mL (5-HTRP) spiked plasma.

Table 4 Concentrations of 5-HT and 5-HIAA in plasma from healthy subjects.

Subject (age, sex)	Concentration (pmol/mL plasma)	
	5-HT	5-HIAA
A (23, male)	15.8	35.8
B (24, male)	15.5	30.8
C (25, male)	16.3	26.2
D (24 female)	24.4	51.9
E (24, female)	11.5	10.8
Mean $\pm$ SD	16.7 $\pm$ 4.71	31.1 $\pm$ 14.9

Figure1

5-HIs	Abbreviation	R
5-Hydroxytryptamine	5-HT	-CH ₂ CH ₂ NH ₂
5-Hydroxyindole-3-acetic acid	5-HIAA	-CH ₂ COOH
<i>N</i> -Acetyl-5-hydroxytryptamine	NAS	-CH ₂ CH ₂ NHCOCH ₃
5-Hydroxytryptophol	5-HTOL	-CH ₂ CH ₂ OH
5-Hydroxytryptophan	5-HTRP	-CH ₂ CH(NH ₂)COOH

Figure2

Figure3