Quantification of Histamine by Postcolumn Fluorescence Detection High-Performance Liquid Chromatography Using Orthophthalaldehyde in Tetrahydrofuran and Reaction Mechanism

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For quantification of histamine, we developed a highly specific and sensitive method of postcolumn fluorescence detection HPLC using a tetrahydrofuran solution of orthophthalaldehyde. Its detection limit was 2–5 pg. This method was applied to the measurement of histamine in human blood and urine samples. Analysis by LC/MS and NMR suggested that the fluorophore in this method has an isoindole structure.

Keywords Histamine, ion-exchange high-performance liquid chromatography, postcolumn, orthophthalaldehyde, tetrahydrofuran, fluorescence detection, fluorophore, liquid chromatography/mass spectrometry, nuclear magnetic resonance

Quantification of histamine in the blood and urine requires sensitive analysis methods because of its low concentration. Enzyme immunoassay and radio immunoassay have been used, but their specificity remains a problem as the antibody used often shows high cross-reactivity with 3-methylhistamine, the main metabolite of histamine *in vivo*. In recent years, high-performance liquid chromatography (HPLC) has been applied to the measurement of histamine. In this method, postcolumn fluorescence detection¹ using a two-step condensation reaction with orthophthalaldehyde (OPA) under alkaline conditions followed by acidic conditions², has been used. Although the specificity of this method for histamine is high, the sensitivity and reproducibility are still problems in terms of the transfer of the reagents and the stability of OPA in aqueous solutions.

We were able to overcome these problems by evaluating the HPLC conditions and developing a method with high sensitivity and reproducibility for measuring histamine in human blood and urine. We decided to use OPA, which is present as the cyclic hemiacetal form in protic solvents such as water and alcohol, but as the aldehyde form in aprotic solvents such as CHCl_3 , CH_3CN and tetrahydrofuran (THF).³ We considered that the reaction of OPA with histamine would proceed primarily *via* the aldehyde form and used OPA solution in an aprotic solvent for the postcolumn reaction. Based on our findings, we propose the structures of the fluorophore and the mechanism of its formation in this reaction.

Experimental

Reagents

Histamine diphosphate and histamine were obtained from Wako Pure Chemicals Industries (Osaka, Japan). The other biogenic amines were of reagent grade. All amines were used as 0.01 M perchloric acid solution. The other reagents and solvents were of commercially available reagent grade, HPLC grade and spectroscopic grade. TLC plate Silica Gel (F-254, 0.25 mm and 0.5 mm thick, E. Merck, Darmstadt, Germany) was used.

Equipment

A pump (L-6200 Intelligent Pump, Hitachi, Tokyo, Japan) connected to a degassing device (Shodex Degas, Showa Denko, Tokyo, Japan), a fluorescence HPLC monitor (RF-535, Shimadzu, Kyoto, Japan), a column oven (CTO-6A, Shimadzu), an auto injector (712 WISP, Waters, Milford, MA, USA) and a data processor (Chromatopak, C-R5A, Shimadzu) were used. The OPA solution was delivered using a precision metering pump (A-60-S, Eldex, San Carlos, CA, USA), and a ninhydrin coil (Waters) was used as the reaction coil. Histamine-OPA products were analyzed using a system consisting of a fluorescence spectrophotometer (F-1000, Hitachi) connected to a chromatograph with a UV detector (Vista 5500, Varian, Palo Alto, CA, USA). UV spectra were measured with this HPLC system connected to a photodiode array detector (990J, Waters) and fluorescence spectra were measured with a fluorescence spectrophotometer (F-4010, Hitachi). LC/MS was

performed using a system consisting of an LC-APCI interface connected to a mass spectrometer (M-2000A, Hitachi), and ^1H -NMR and ^{13}C -NMR using an XL-400 and a UNITY-600 (Varian).

Measurement

HPLC conditions. A TSKgel Catecholpak (6.0 mm i.d. $\times$ 15 cm, TOSOH, Tokyo, Japan) was used as the column. The column temperature was 40°C, and 0.2 M propionate buffer (pH 4.5) containing 0.086 M NaCl as the eluent was delivered at a flow rate of 1.0 ml/min. As the postcolumn reagent, THF solution of 0.1% OPA was delivered at a flow rate of 0.6 ml/min. The reaction temperature was 40°C, and detection was done at an excitation of 360 nm and an emission of 430 nm. Primary amines, such as polyamine, catecholamine, and amino acids, were measured by the postcolumn detection method using 2-mercaptoethanol and OPA.⁴

Preparation of samples. Serum samples (1 ml) were mixed with 0.6 ml of 10% trichloroacetic acid (TCA) and centrifuged at 10500g for 2 min. The supernatant (0.1 ml) was subjected to the HPLC. Similarly, urine samples were diluted tenfold with distilled water and analyzed by HPLC.

Reaction mechanism

HPLC conditions. For structure analysis of the histamine-OPA reaction product (fluorophore), a Capcellpak C₁₈ SG-120 (5 μm , 4.6 mm i.d. $\times$ 25 cm, Shiseido, Tokyo, Japan) was used as the column, and 0.05 M ammonium acetate/ CH_3CN (gradient elution from 40% CH_3CN to 80% CH_3CN during 20 min) was delivered as the eluent at a flow rate of 1.0 ml/min. For detection, UV (254 nm and 360 nm) and fluorescence (Ex 360 nm/Em 430 nm) were used. LC/MS was performed under both the same reversed-phase HPLC conditions and the normal-phase HPLC conditions [column, Lichrosorb Si-60 (5 μm , 4.0 mm i.d. $\times$ 12.5 cm, Kanto Chemical, Tokyo, Japan); eluent, ethyl acetate/ CH_3OH 95:5 (v/v); flow rate, 1.0 ml/min]. Stable nonfluorescent products considered to be derived from the fluorophore were separated using a Develosil 5C₁₈ (5 μm , 4.6 mm i.d. $\times$ 15 cm and 10 mm i.d. $\times$ 25 cm, Nomura Chemical, Seto, Japan) and 0.2% TFA aqueous solution/ CH_3CN (gradient elution from 10% CH_3CN to 50% CH_3CN during 20 min) as the eluent was delivered at 1.0 ml/min (analysis) or 5.0 ml/min (preparation). UV (254 nm) was used for detection.

Sample preparation. For the analysis of the histamine-OPA reaction product (fluorophore), 10 ml of 0.2 M propionate buffer (pH 7.0) containing 2.3 mM histamine was mixed with 5 ml of THF solution of OPA (mol ratio of 1, 2, 10 and 20 to histamine), and the reaction mixture was left at room temperature for 2 min. An aliquot of the reaction mixture was subjected to the HPLC. For LC/MS, a similar procedure was performed with the same buffer containing 11.5 mM histamine and OPA (mol ratio of 1, 2 and 5 to histamine). The stable nonfluorescent products were extracted with

40 ml of benzene after 20 ml of a THF solution of 1.2 g OPA was added to 40 ml of 0.2 M propionate buffer (pH 7.0) containing 0.2 g of histamine and the mixture had been left standing for 1 min at room temperature. The extract was dried with magnesium sulfate, and the solvent was removed under reduced pressure. The residue was separated by TLC [ethyl acetate/ CH_3OH , 5:2 (v/v)] and then purified by HPLC. One powdered product (D1) and two oily products (D2 and D3) were obtained by freeze-drying of HPLC fractions.

NMR analysis of the fluorophore. THF- d_8 solution (7.5 ml) containing 25 mg of OPA was mixed with 15 ml of 0.2 M propionate buffer (pH 7.0) containing 80 mg of histamine. After 1 min, the reaction mixture was extracted with 10 ml of CCl_4 . Magnesium sulfate and 0.1 ml of acetic acid- d_4 were added to the extract. After 2 h at -20°C, the dehydrated extract was analyzed with NMR.

Results and Discussion

Measurement

Table 1 shows the fluorescence intensity (relative peak height) detected by the postcolumn addition of the OPA solution prepared using various solvents. The fluorescence intensity was higher for aprotic solvents such as DMF, dioxane, CH_3CN , and THF than for protic solvents such as water and alcohol. The highest fluorescence intensity was observed for reagent grade THF containing 0.025% of 2,6-di-*t*-butyl-*p*-cresol (BHT) as a stabilizer. These findings suggest that OPA reacts mainly with histamine in the aldehyde form. Hayashi⁵ reported a postcolumn detection method of histamine with OPA solution using an acid-alcohol mixture 1:1 (v/v) as the solvent. However, this HPLC assay must be run under strict conditions, e.g., those related to pH and salt concentration of eluent.

When the concentration of the OPA solution in THF was changed stepwise from 0.05 to 2.0%, the maximum fluorescence intensity was observed at the OPA concentration of 0.1%. The optimum flow rate of the OPA solution was 0.6–0.7 ml/min. As for the reaction

Table 1 Effect of several solvents for OPA solution (0.1%) on relative peak height

Solvent	Relative peak height
Water	1
Methanol	7
Ethanol	23
Acetone	7
Dimethylformamide	52
Dioxane	61
Acetonitrile	34
Tetrahydrofuran (HPLC)	85
Tetrahydrofuran (R.G.)	100

Sample (histamine solution, 1.1 μM , 50 μl) was injected into the HPLC.

Table 2 Capacity factor and relative response of histamine and biogenic amines obtained by the present HPLC method

Amine	k'	Relative response
Histamine	3.20	100
1-Methylhistamine	3.17	N.D
3-Methylhistamine	3.17	N.D
3-Methylhistidine	0.42	N.D
Histidinol	2.97	75
Serotonine	6.83	0.06
Catecholamine	2.11	N.D
Amino acids ^a	<1.00	N.D
Histidine		59
Tryptophan		0.5
Putrescine	2.76	N.D
Cadaverine	2.77	N.D
Spermidine	10.00<	N.D
Spermine	10.00<	N.D
<i>N</i> -Acetylputrescine	0.58	N.D
<i>N</i> -Acetylcadaverine	0.66	N.D
<i>N</i> -Acetylspermidine	2.28	N.D
<i>N</i> -Acetylspermine	2.45	N.D
<i>N</i> -Acetylspermine	10.00<	N.D

a. L-lysine, L-arginine, L-aspartic acid, L-threonine, L-serine, L-glutamic acid, L-proline, L-isoleucine, L-alanine, L-cystine, L-valine, L-methionine, glycine, L-leucine, L-tyrosine, L-phenylalanine, L-tryptophan.

temperature, the fluorescence intensity at 40–50°C was about 1.3 times that at room temperature. Although addition of BHT to THF increased the fluorescence intensity, the greater the BHT concentration, the more noise there was on the chromatogram. Therefore, reagent grade THF containing 0.025% BHT was used without any treatment. The mechanism of the increase in fluorescence intensity by BHT is not clear.

A good calibration curve passing the origin was obtained between the peak height on the chromatogram and the amount of histamine in the range of 10–1280 pg ($r=0.999$, $n=8$). The reproducibility (RSD) of the peak height on serial analysis for more than 20 h was 3.2–4.5% (concentration level: 10, 20, 40, 80, 160, 320, 640 and 1280 pg; $n=9$). Therefore, the absolute calibration method using the peak height was considered to be applicable to the quantification. The lower detection limit was 5 pg ($S/N=3$).

Fig. 1 HPLC chromatograms of serum samples: (a) blank serum; (b) serum spiked with histamine (11 μM). Deproteinized serum (50 μl) was injected into the HPLC.

The capacity factor (k') and the fluorescence intensities for histamine and various other biogenic amines are shown in Table 2. As presumed from k' , histamine can be distinguished from the other amines, except methylhistamine, by this method. As for methylhistamine, it will not interfere with the measurement because it produces no fluorophore under the reaction conditions. As shown in Table 2, the present method allows selective detection of compounds with a β -aminoethyl group at the β -position of the imino group on imidazole and indole such as histamine and histidine. Although Shore's method is similar to the present method, the former detects spermidine as well as histamine and histidine.

Recovery test

Histamine in human serum. Figure 1 shows chromatograms of pooled serum and histamine-spiked pooled serum. Histamine was clearly separated by the analysis within 20 min, and the lower quantification limit was 1 nM. The recovery of histamine from histamine-spiked serum in the range of about 7–110 nM was 104–114% as shown in Table 3. Though these values were slightly higher than the theoretical value, there was a good linear relationship between the spiked amount and the observed value. The intra-day and inter-day

Table 3 Recovery of histamine from human serum

Sample No.	Added (X)/nM	Found (Y)/nM						Mean $\pm$ SD	Recovery, % Mean $\pm$ SD
1–5	0	3.5	3.8	3.1	3.8	3.2		3.5 $\pm$ 0.3	
6–10	7.0	11.6	11.2	11.7	11.6	11.3		11.5 $\pm$ 0.2	114.3 $\pm$ 5.7
11–15	13.9	19.4	19.1	19.2	18.5	18.9		19.0 $\pm$ 0.3	111.5 $\pm$ 3.6
16–20	27.8	34.7	34.3	33.9	34.0	34.0		34.2 $\pm$ 0.3	110.4 $\pm$ 1.4
21–25	55.6	63.6	65.5	63.8	64.5	66.6		64.8 $\pm$ 1.1	110.3 $\pm$ 2.2
26–30	111.2	118.7	118.6	118.7	122.0	117.9		119.2 $\pm$ 1.4	104.0 $\pm$ 1.2

Regression equation: $Y=1.042X+4.6$, $SD=1.48$, $r=0.9994$.

Fig. 2 HPLC chromatograms of urine samples: (a) blank urine; (b) urine spiked with histamine (1.4 μ M). Deproteinized sample of diluted urine was injected into the HPLC.

precision values for serum samples containing 27.8 nM of histamine were 0.8% ($n=5$) and 3.3% ($n=5$), respectively. **Histamine in human urine.** Figure 2 shows chromatograms of pooled urine and histamine-spiked pooled urine. Since many interference peaks were observed, unlike blood samples, the urine samples need to be diluted tenfold to reduce the interference. The lower quantification limit was 10 nM. Table 4 shows the recovery of histamine from urine samples. The recovery of histamine in the range of about 170–2780 nM was 99–102%. There was a good linear relationship between the spiked amount and the observed value ($Y=1.00X$). The intra-day and inter-day precision values for urine samples containing 694 nM of histamine were 0.9% ($n=5$) and 2.0% ($n=5$), respectively.

Improvement in sensitivity

Although the above method showed high sensitivity for histamine, we tried to improve it further. Figure 3 shows the time course of fluorescence intensity of the reaction mixture of 0.2 M propionate buffer solution of histamine (pH: 3.5, 4.0, 4.5, 5.0, 5.5, 6.4, 6.8, 8.3) with THF solution containing 0.1% OPA. At each pH, the fluorescence intensity reached the maximum after 3 min

Fig. 3 Time course of fluorescence intensity at different pH. pH: (a) 3.5; (b) 4.0; (c) 4.5; (d) 5.0; (e) 5.5; (f) 6.4; (g) 6.8; (h) 8.3. As the control, the fluorescence intensity at pH 8.3 was used and measured at Ex 360 nm/Em 430 nm. A histamine concentration, 55.5 μ M.

and decreased thereafter. The fluorescence intensity of this reaction depended on pH, reaching a maximum at pH 7–8. The half-life of fluorescence was estimated to be 15–20 min. Therefore, after alkaline solution was added to the column eluate of this HPLC system to adjust the pH to about 7, THF solution containing 0.1% OPA was delivered (2-step postcolumn reaction). Compared with the original method, the sensitivity increased about twofold, and the lower detection limit became 2 pg.

Reaction mechanism

The HPLC chromatogram of the reaction mixture of 0.2 M propionate buffer containing histamine and THF solution of OPA shows two fluorescent peaks (P1, P2). P1 decreased and P2 increased with an increase in the OPA concentration. Reversed-phase LC/MS of P1 showed ions at m/z 228 [$M+H_2O+H$] $^+$, 210, 182, 146, 116, 95, 81 and 68, and that of P2 showed ions at m/z 362 [$M+H_2O+H$] $^+$, 344, 326, 231, 210, 135, 116, 95 and 68. In addition, normal-phase LC/MS of P1 showed ions at m/z 210 [$M+H$] $^+$, 182, 116 and 81. These results suggested that this reaction initially produces a 1:1

Table 4 Recovery of histamine from human urine

Sample No.	Added (X)/nM	Found (Y)/nM						Mean $\pm$ SD	Recovery, % Mean $\pm$ SD
1–5	0	140	152	148	176	188		161 $\pm$ 18	
6–10	173	317	337	337	340	360		338 $\pm$ 14	102.3 $\pm$ 5.2
11–15	347	490	508	524	525	530		515 $\pm$ 15	102.3 $\pm$ 3.5
16–20	694	894	840	867	867	867		867 $\pm$ 17	101.7 $\pm$ 3.9
21–25	1388	1520	1533	1591	1603	1621		1573 $\pm$ 40	101.8 $\pm$ 1.9
26–30	2775	2907	2910	2993	2917	2873		2920 $\pm$ 40	99.4 $\pm$ 1.9

Regression equation: $Y=0.995X+171$, SD=13.6, $r=0.9999$.

Fig. 4 Excitation and emission spectra of the histamine-OPA reaction mixture. (a) P1-rich mixture; (b) P2-rich mixture.

Fig. 5 UV spectra of histamine-OPA reaction products.

histamine-OPA addition product (P1, fluorescent, intermediate) and then a 1:2 histamine-OPA addition product (P2, fluorescent). P2 or the mixture of P1 and P2 is considered to be the fluorophore detected by HPLC. The fluorescent spectra of P1-rich and P2-rich mixture are shown in Fig. 4, and their UV spectra, obtained using a photodiode array detector, in Fig. 5.

P1 was extracted with CCl_4 from the cooled reaction mixture together with other minor components and was measured by NMR. NOE correlation and spin-spin coupling correlation indicated the structure of the major component P1 to be 4, as seen in Fig. 6. NMR analysis revealed that the structures of the nonfluorescent products are 10 and 11 (Fig. 6). The NMR data proving the structures are shown in Fig. 7. D1 was the stereoisomer 10a. D2 was a mixture of 10b and 10c (2:3). D3 was a mixture of 11a and 11b (7:5). These results led to the reaction course shown in Fig. 6. First, condensation between histamine and OPA produces

cyclic intermediate 1. This intermediate is dehydrated, producing fluorophore 2, which is considered to be the intermediate in this reaction. [Compound 4 is considered to be produced from intermediate 2 after ring-closing dehydration. However, since ions at m/z 228 $[\text{M}+\text{H}_2\text{O}+\text{H}]^+$ and m/z 210 $[\text{M}+\text{H}]^+$ were obtained by reversed-phase LC/MS and normal-phase LC/MS, respectively, a reversible reaction involving H_2O may occur between 3 and 4.] Next, another molecule of OPA is added to 2, producing fluorophore 6 via keto-form tautomer 5 (the presence of the determined structures 10 and 11 in Fig. 6 supports the structure and presence of 6). This structure 6 probably produces fluorophore 8 or 9 via compound 7, although clear evidence is lacking to decide between structures 8 or 9. These results suggest that P1 has the structure of 2 or 4, and P2 of 6 or perhaps of 8 or 9. The structures 2, 4, 6, 8 and 9 are the isoindole type. Studies on primary amines and OPA in the presence of the thiol component showed marked fluorescence due to the isoindole structure.⁶⁻⁸ Rönnerberg *et al.*⁹ reported production of a histamine-OPA (1:1) addition product, followed by production of the 1:2 product. However, their estimated structures differed from ours. After treatment with a strong alkali followed by a strong acid, the 1:2 product gave a stable substance emitting intense fluorescence. In our method, the reaction is done in neutral solution, and both the 1:1 and 1:2 products are fluorescent with the same fluorescence spectra. These addition products lost their fluorescence under strong acidic condition, being rapidly degraded. Therefore, the fluorescence substance obtained in this study probably differs from that reported by Shore *et al.* or Rönnerberg *et al.*

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Fig. 6 Proposed mechanism of the fluorescence reaction of histamine with OPA.

(4) ¹H Data (in CCL₄-THF-CD₃COOD at 20°C)

NOE

(10: three stereoisomers a, b, and c)

¹H Data (in CD₃OD at 20°C) ¹³C Data (in CD₃OD at 28°C)

(11: two geometric isomers a and b)

 ^1H Data (in Acetone- d_6 at 20°C) ^{13}C Data (in Acetone- d_6 at 20°C)

Fig. 7 NMR data and NOE correlation of products 4, 10 and 11.

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