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High-performance liquid chromatographic method for rapid and highly sensitive determination of histidine using postcolumn fluorescence detection with *o*-phthaldialdehyde

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Abstract

A high-performance liquid chromatographic method was developed for the rapid and sensitive determination of histidine. The method is based on separation by reversed-phase ion-pair chromatography followed by highly selective fluorescence derivatization of histidine with *o*-phthaldialdehyde. A linear calibration curve was obtained over the range of 0.25–200 pmol per injection (10 μl) with the coefficient of variation of 0.9% at 2 pmol ($n=10$) and with the detection limit ($S/N=8$) of 25 fmol. The method was applicable to the assay of histidine in human serum. Serum histidine values obtained by the present method were in good agreement with values obtained with an amino acid analyzer.

Keywords: *o*-Phthaldialdehyde; Histidine

1. Introduction

o-Phthaldialdehyde (OPA) is known to react with primary amines in the presence of thiol such as 2-mercaptoethanol, to form a highly fluorescent adduct [1–3]. This fluorescence reaction has been widely used for the detection of amines and amino acids in chromatographic systems [4–8]. In the absence of a thiol, OPA can react with histidine [9–11], histamine [12–20], agmatine [21], spermidine [22,23], serotonin [24] and glutathione [25,26] to give fluorescent adducts under the appropriate reaction conditions such as pH, and has been used as a selective fluorogenic reagent for the assay of these biogenic amines and amino acids in biological samples. In the case of histidine [11], it reacts gradually with OPA alone in an alkaline medium to give a relatively stable fluorescent adduct showing excitation and emission maxima at 360 and 440 nm, respectively. Håkanson et al. [11] optimized these reaction conditions and showed that the fluorescence intensity due to histidine reached maximum 10 min after initiation of the reaction at pH 11.2–11.5 and at 40°C.

On the other hand, we revealed the mechanistic pathways of the OPA-induced fluorescence reaction

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of histamine [27] and of histidine [28], and have recently found that the fluorescent adduct of histidine forms rapidly in a neutral or weakly alkaline media though its stability is low. These findings led us to investigate the reaction conditions optimal for a postcolumn fluorescence detection of histidine in its high-performance liquid chromatography (HPLC). Though the reagent system of OPA and a thiol can be also used for postcolumn detection of histidine, it detects all commonly encountered other amino acids and primary amines in biological samples [1–3], so that a precise separation of histidine from these substances is required prior to the postcolumn detection [4–8]. In contrast, the fluorescence reaction with OPA alone is selective for histidine and is scarcely interfered with by other amino acids [11], allowing the separation conditions to be quite simple. The present paper describes HPLC method for the specific and sensitive determination of histidine with this postcolumn fluorescence detection and its application to the assay of histidine in human serum.

2. Experimental

2.1. Chemicals.

L-Histidine monohydrochloride, histamine dihydrochloride, carnosine, glutathione, putrescine dihydrochloride, 5-sulfosalicylic acid (5-SSA) and OPA were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 1-Methyl-L-histidine and 3-Methyl-L-histidine were obtained from Hoechst Japan Ltd. (Tokyo, Japan). L-Amino acids were from Ajinomoto Company (Tokyo, Japan) and spermidine was from Nakarai Chemicals (Kyoto, Japan). Sodium octanesulfonate and agmatine sulfate were purchased from Tokyo Kasei Organic Chemicals (Tokyo, Japan). HPLC-grade methanol was obtained from Kanto Chemical (Tokyo, Japan). All other reagents used were of analytical grade. Milli-Q (Nihon Millipore Kogyo, Yonezawa, Japan) water was used in the preparations of an eluent and reagent solutions. A stock standard solution of histidine (20 m*M*) was prepared in 0.02 *M* HCl and stored in a refrigerator. Working standard solutions of histidine were prepared by diluting the stock solution with 0.02 *M* HCl before use. OPA solution was freshly prepared by dissolving in methanol.

2.2. Equipment

Fluorescence measurements were made with a Shimadzu RF-500 fluorescence spectrophotometer using 10 mm-diameter cuvettes thermostated at 40°C. For centrifugation of human serum samples, a Kubota Model 1900 micro refrigerated centrifuge (Tokyo, Japan) was used. Chromatographic experiments were performed using a Shimadzu (Kyoto, Japan) HPLC system including two LC-9A pumps for delivery of an eluant and OPA reagent, a CTO-6A column oven, a RF-10A fluorescence detector equipped with a 15 μl square flow cell, and a CR-6A data processor. A Rheodyne Model 7125 with a 20 μl sample loop (Rheodyne, Cotati, CA, USA) was used as a sample injector. Elutions of underivatized histidine, other amino acids and amines from HPLC column were monitored at 220 nm by using a Shimadzu SPD-10AV absorbance detector. Amino acid analyses of serum samples were performed with Hitachi Model L-8500 amino acid analyzer (Tokyo, Japan), in which amino acids were separated by ion-exchange chromatography and detected with ninhydrin.

2.3. Fluorescence reaction of histidine with OPA

To 3.0 ml of 50 m*M* sodium phosphate buffer (pH 6.5–11.5) preincubated in a cuvette thermostated at 40°C was added 100 μl of 0.1 m*M* standard histidine solution and the reaction was initiated by adding 100 μl of 50 m*M* OPA solution. The final concentrations of histidine and OPA were 1.56 μ*M* and 3.2 m*M*, respectively. The fluorescence intensity at 435 nm was monitored periodically with an excitation wavelength of 365 nm. The intensity was corrected against the reagent blank which corresponded to the intensity given by about 20 n*M* histidine.

2.4. Preparation of human serum sample

A hundred microliters of human serum (stored at −80°C before use) or control serum (Wako Pure Chemical Industries Ltd., Osaka, Japan) was mixed with 100 μl of 2% (w/v) 5-SSA in a 1.5 ml

Eppendorf polypropylene micro test tube and was shaken for 15 min. The mixture was centrifuged at 16 000×*g* for 15 min at 4°C, then the supernatant was diluted 100 times with 0.02 *M* HCl and was filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA). A portion (10 μl) of the filtrate was directly injected onto the HPLC column. For the determination of histidine with the amino acid analyzer, the same supernatant was diluted four times with 0.02 *M* HCl and filtered through a 0.45 μm membrane filter. A portion (100 μl) of the filtrate was injected into the amino acid analyzer.

2.5. Chromatographic system and conditions

Separation was performed at 40°C by using a 30×4.6 mm I.D. Develosil ODS UG-3 column with a particle size of 3 μm (Nomura Chemical, Seto, Japan) as an analytical column which was protected by a guard-pak cartridge column (a 10×4.0 mm I.D. Develosil ODS UG-5, particle size of 5 μm), and a 5:95 (v/v) mixture of methanol and sodium phosphate buffer (35 m*M*, pH 6.2) containing 5.3 m*M* sodium octanesulfonate as an eluent. OPA reagent used for postcolumn detection of histidine was a 15:1 (v/v) mixture of 50 m*M* sodium phosphate buffer (pH 8.0) and 50 m*M* OPA in methanol. Both the eluent and OPA reagent were filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA) and degassed ultrasonically before use. The eluent was delivered to the column at a flow-rate of 0.5 ml min^{-1} through a preheater tube (stainless steel tube, 10 m×0.8 mm I.D) and the injector. Ten microliters of sample solution was introduced to the column *via* the sample injector with 10 μl microsyringe. The eluate from the column was added with OPA reagent delivered at a flow-rate of 0.5 ml min^{-1} to a mixing T-joint attached to the column through a preheater tube (stainless steel tube, 10 m×0.8 mm I.D) and a resistor polytetrafluoroethylene (PTFE) tube (20 m×0.25 mm I.D). The mixture was passed through a reactor tube (coiled PTFE tube, 2.5 m×0.5 mm I.D, coil diameter of 20 mm) and the generated fluorescence was detected at 435 nm with an excitation wavelength of 365 nm (uncorrected). All columns, preheater-, resistor- and reactor tubes were placed in the column oven maintained at 40°C.

Specificity of the postcolumn fluorescence detection with OPA (Table 1) was tested by using this chromatographic system, except that a PTFE resistor tube (20 m×0.25 mm I.D) was used instead of the columns (flow method).

Table 1
Relative fluorescence intensity (RFI) in the fluorescence detection with *o*-phthaldialdehyde and retention time (Rt) of biological substances

Substance	RFI (%)[a]	Rt (min)[b]
L-Histidine	100	2.7
Histamine	14.4	51
1-Methyl-L-histidine	<0.01	3.3
3-Methyl-L-histidine	<0.01	2.7
Carnosine	<0.15	4.4
Glutathione	11.8	1.4
Agmatine, putrescine, spermidine	<0.01	>80
Ornithine	<0.01	3.9
A mixture of 19 amino acids	1.2	
Each of 19 amino acids		
Asp, Glu,	<0.01	1.4
Asn, Gln, Ser, Ala, Thr, Gly, Cys	<0.01	1.6
Pro, Val, Met, Tyr	<0.01	1.7–2.1
Ile,	<0.01	2.5
Leu	<0.01	2.7
Trp	1.2	6.9
Lys, Phe, Arg	<0.01	4.0–7.8

[a] Fluorescence intensity was measured by flow method as described in Section 2.5 and corrected on the molar basis. Injected amounts were 5 pmol for histidine, 20 pmol for histamine and glutathione, 400 pmol for putrescine, 2 nmol for 1-methylhistidine and 3-methylhistidine, and 200 pmol for other biological substances. Since carnosine, 1-methylhistidine and 3-methylhistidine were shown to contain histidine as an impurity at the levels of 0.27, 0.04 and 0.012%, respectively, the intensity due to the impurity was subtracted from the total apparent intensity.
[b] Elution of the biological substances was monitored at 220 nm.

3. Results and discussion

3.1. Fluorescence reaction of histidine with OPA

First, the effect of pH (6.5–11.5) on the time course of the OPA induced fluorescence reaction was examined in the batch method in order to optimize its reaction conditions for postcolumn detection of histidine. In these experiments, the concentration of OPA and reaction temperature were set at 1.56 m*M* and at 40°C, respectively, after prelinary investigations. As shown in Fig. 1, both the rates of formation and degradation of the fluorescent adduct of histidine

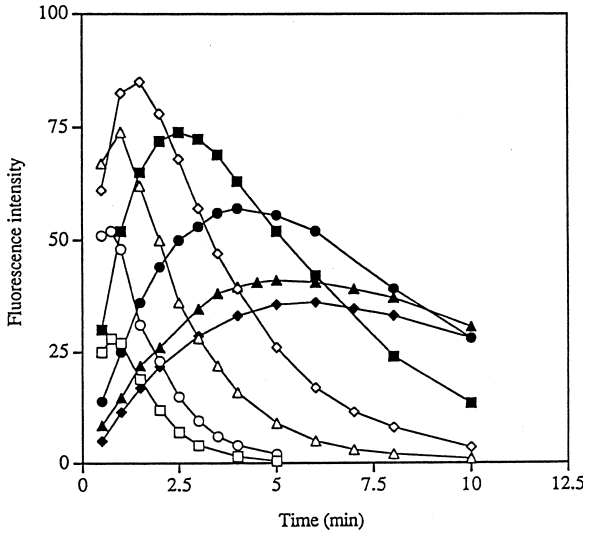

Fig. 1. Effect of pH on the time course of the fluorescence reaction of histidine with *o*-phthaldialdehyde at 40°C. The pHs of the reaction mixtures were 6.5 (□), 7.0 (○),7.5 (△), 8.0 (◇), 9.0 (■), 10.0 (●), 11.0 (▲) and 11.5 (♦), respectively. See Section 2.3 for other reaction conditions.

increased with the decrease of pH, and the highest fluorescence intensity was obtained at pH 8.0. However, judging from the reaction time required to reach a maximal fluorescence intensity, a pH around 7.0 seemed to be optimal where the maximal intensity was obtained in one minute.

3.2. Separation conditions

As described below in detail, this fluorescence reaction was highly selective for histidine. Since glutathione seemed to be the sole interfering substance, we had only to separate glutathione from histidine prior to the fluorescence reaction. The separation was easily achieved by a reversed-phase ion-pair chromatography on an ODS short column with a 5:95 (v/v) mixture of methanol and sodium phosphate buffer (35 m*M*, pH 6.2) containing 5.3 m*M* sodium octanesulfonate at a flow-rate of 0.5 ml min^{-1}. Under these conditions histidine and glutathione were eluted at 2.1 min and 0.9 min, respectively (monitored at 220 nm).

3.3. Conditions for postcolumn fluorescence detection of histidine with OPA

Since the reaction temperature markedly influences the rates of formation and degradation of the fluorescent adduct of histidine, its precise control is an essential factor for reproducibility of the postcolumn fluorescence detection. Therefore, preheatments of both the eluent and OPA reagent to a constant temperature of 40°C were required before their mixing, and these were achieved by insertion of the preheater tubes for both the eluent and OPA reagent into the line. As described in Section 2 the preheater tubes as well as the columns, resistor tube and the reactor tube were placed in a column oven maintained at 40°C. In these experiments, optimal conditions for the postcolumn detection of histidine were investigated by using this system, except that a PTFE resistor tube (20 m×0.25 mm I.D) was used instead of the columns. With the flow-rate of the eluent (pH 6.2) being fixed at 0.5 ml min^{-1}, the effects of pH and reaction time were examined by changing the flow-rate (0.2–0.8 ml min^{-1}) of OPA reagent (in 50 m*M* sodium phosphate buffer, pH 8.0) and the length of reactor tube. As a result, the reactor tube of 2.5 m was chosen and the flow-rate of OPA reagent was set at 0.5 ml min^{-1}, judging from the fluorescence intensity and its reproducibility. Under these conditions, final pH of the reaction mixture was shown to be 7.07.

3.4. Specificity of the postcolumn fluorescence detection with OPA

Specificity of the postcolumn fluorescence reaction with OPA was tested under the conditions established above (flow method) and shown in Table 1. No significant fluorescence (peak intensity) was observed with derivatives of histidine such as 1-methylhistidine, 3-methylhistidine and carnosine (β-alanyl-L-histidine), and with biogenic amines such as agmatine, putrescine and spermidine. Amino acids except for histidine and tryptophan gave no detectable fluorescence. Though tryptophan gave fluorescence, the intensity was only 1.2% of that given by histidine on a molar basis. Similar weak fluorescence intensity (1.2%) was also obtained with a mixture of 19 amino acids, which was likely due to tryptophan.

Furthermore, the intensity given by histidine increased by the coexistence of the mixture of 19 amino acids, however, the increases were only 2.0 and 3.5% at an equal- and 2-fold amount of each of the amino acids, respectively. Therefore, the interference from amino acids with this postcolumn fluorescence detection seemed to be quite low or negligible.

Of the biological substances tested, histamine and glutathione showed significant fluorescence. Though histamine reacts with OPA similarly to histidine [27,28], its fluorescence intensity was 14.4% of that given by histidine because the fluorescent adduct of histamine was less stable than that of histidine [28]. Most biological samples normally contain large excess amount of histidine to histamine [6,18,19,29,30], especially human serum contains histidine at level 1000-fold higher than that of histamine [9,19]. Thus, the interference from histamine seems to be negligible when the postcolumn fluorescence detection is applied to the assay of histidine in such biological samples.

The fluorescence intensity of glutathione was 11.8% of that given by histidine, which was similar to that of histamine, however, the levels of glutathione are comparable to or higher than those of histidine in various biological samples such as livers, kidneys and blood (mainly in erythrocytes) [31]. Thus, glutathione is not negligible as an interfering substance when it is coeluted with histidine.

3.5. HPLC method with postcolumn fluorescence detection

A highly sensitive method for the determination of histidine was developed using reversed-phase ion-pair chromatography in conjunction with the post-column fluorescence detection described above. Fig. 2A shows a typical chromatogram of a mixture of standard histidine and glutathione. The peaks due to glutathione and histidine were observed at 1.4 and 2.7 min, respectively. The chromatogram was not essentially affected by coexistence of the 19 amino acids in the standard mixture though leucine and isoleucine were almost coeluted with histidine at 2.5–2.7 min (monitored at 220 nm) as shown in Table 1. Under this condition, histamine and tryptophan was eluted at 51 and 6.9 min, respectively.

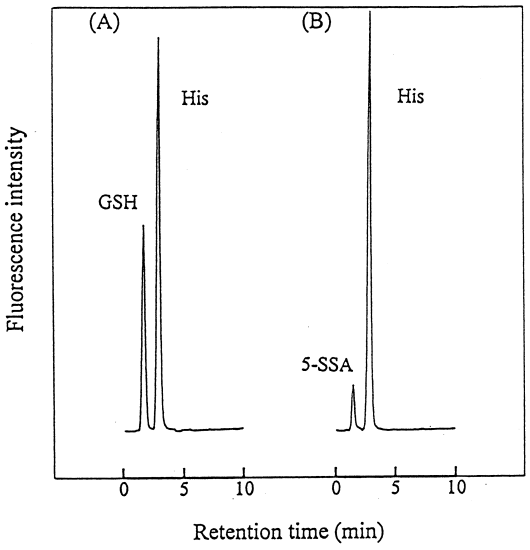

Fig. 2. Typical chromatograms of histidine. (A) Standard histidine and glutathione. Injected amounts: histidine (His), 4 pmol; glutathione (GSH), 20 pmol. (B) Human serum sample after deproteinization with an equal volume of 2% (w/v) 5-sulfosalicylic acid (5- SSA) followed by dilution 100 times with 0.02 *M* HCl. Injected volume was 10 μl. See Section 2.5 for chromatographic conditions.

Fig. 3 shows the linearity and the reproducibility of the present HPLC method. A linear calibration curve ($r=1.000$) was obtained over the range of 0.25–200 pmol per injection (10 μl) with the coefficient of variation of 0.9% at 2 pmol ($n=10$) and with the detection limit ($S/N=8$) of 25 fmol.

3.6. Application to human serum sample

As an application of the present HPLC method to a biological sample, we determined histidine in human serum. Fig. 2B shows the typical chromatogram of human serum after deproteinization with 5-SSA followed by dilution with 0.02 *M* HCl. The high selectivity of the post column detection made the chromatogram quite simple as shown in Fig. 2B where the peak due to histidine appeared at 2.7 min with the peak due to 5-SSA at 1.4 min. Though glutathione was coeluted with 5-SSA under this condition, it could scarcely be detected because its

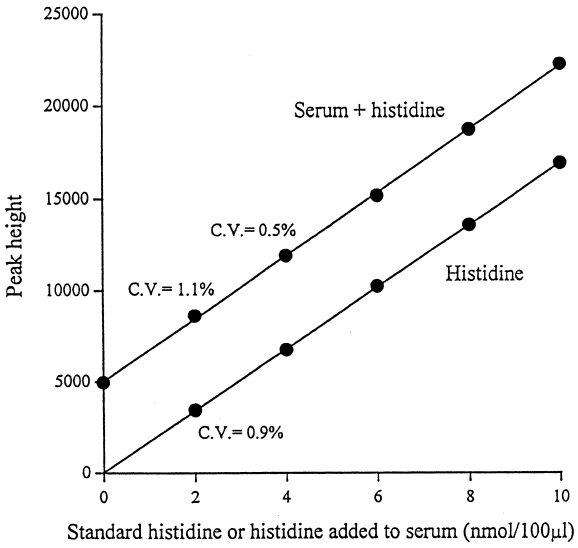

Fig. 3. Peak height as a function of the amount of histidine in standard solution and histidine added to human serum (2–10 nmol/100 μl of serum). See Section 2.4 for serum sample preparation and Section 2.5 for chromatographic conditions.

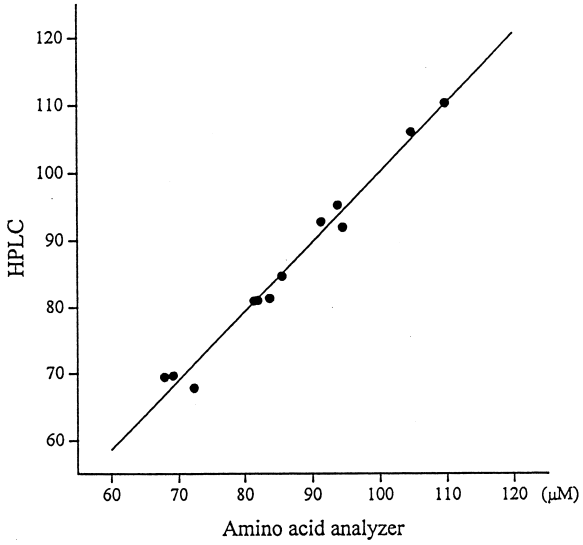

Fig. 4. Correlation between histidine values in human sera ($n=12$) determined by the present HPLC method (y) and by amino acid analyzer (x). The linear regression equation is $y=1.037x-3.650$ (correlation coefficient=0.991). See Section 2.4 for serum sample preparation and Section 2.5 for chromatographic conditions.

serum level (2.7–4.2 μM) [32–34] is 20–30 fold lower than that of histidine.

Linearity and recovery of the present method were tested by using control sera to which were added various amounts of standard histidine (2–10 nmol per 100 μl of serum) prior to sample preparation. As shown in Fig. 3, the peak intensity was found to be a linear function of the amount added histidine with a slope closely similar to that obtained from standard histidine. The mean recovery of histidine added to the sera was determined from the slope ratio to be 101.7% with the coefficients of variation ($n=10$) of 1.1% at 2 nmol/100 μl and 0.5% at 4.0 nmol/100 μl of serum, respectively.

The correlation between the present HPLC method and an amino acid analyzer was examined by assays of human sera ($n=12$) after deproteinization with 5-SSA followed by dilution. As shown in Fig. 4, there was a satisfactory linear correlation between the two methods, and the linear regression equation was calculated as $y=1.037x-3.650$ ($r=0.991$).

The mean value of histidine in sera from healthy volunteers ($n=30$) was determined by the present method to be 89.5±12.6 μM (mean±S.D.). The coefficient of variation of the present method was also examined by using human pooled serum. The within-run variation was 0.59% ($n=10$) and the day-to-day variation was 0.78% ($n=50$). Because of high sensitivity of the postcolumn detection, the present method permits quantitative determination of histidine in serum even diluted by a factor of about 4000.

Though numerous methods are now available for the determination of histidine, most of them are HPLC methods including amino acid autoanalyzers, which are developed for amino acid analysis with pre- or postcolumn chemical derivatization selective for a primary amine [35]. These methods are sensitive for histidine and afford useful information on other amino acids, however, they are time-consuming and inadequate for the assay of histidine in a large number of biological samples. Although several spectrophotometric [36–38] and fluorometric [11] batch methods have been reported for the assay of histidine, their selectivities for histidine are low and many biological substances interfere with the assays. On the other hand, the enzymatic method can be used for the specific assay of histidine in biological samples, which depends on spectrophotometric determination of urocanic acid formed from histidine

by histidine ammonia-lyase [39,40]. However, its sensitivity is lower than that of the present method by a factor of 10^5. Therefore, the present method has several advantages over the reported methods such as specificity, high sensitivity, reproducibility and rapidity for the determination of histidine in various biological samples.

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