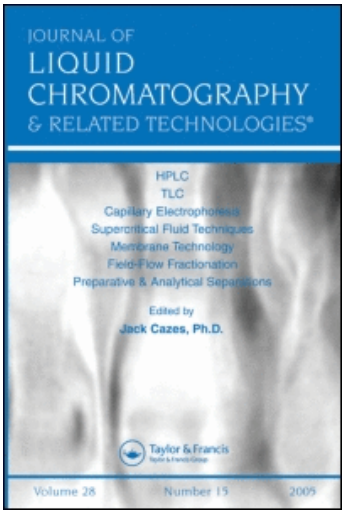

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 31 December 2001

To cite this Article Tateda, Naohiro , Matsuhisa, Kiichi , Hasebe, Kiyoshi and Miura, Toshiaki(2001) 'SENSITIVE AND SPECIFIC DETERMINATION OF HISTIDINE IN HUMAN SERUM, URINE, AND STRATUM CORNEUM BY A FLOW INJECTION METHOD BASED ON FLUORESCENCE DERIVATIZATION WITH o-PHTHALALDEHYDE', Journal of Liquid Chromatography & Related Technologies, 24: 20, 3181 — 3196
To link to this Article: DOI: 10.1081/JLC-100107728
URL: http://dx.doi.org/10.1081/JLC-100107728

J. LIQ. CHROM. & REL. TECHNOL., 24(20), 3181–3196 (2001)

SENSITIVE AND SPECIFIC DETERMINATION OF HISTIDINE IN HUMAN SERUM, URINE, AND STRATUM CORNEUM BY A FLOW INJECTION METHOD BASED ON FLUORESCENCE DERIVATIZATION WITH o-PHTHALALDEHYDE

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ABSTRACT

Flow injection method was developed for rapid, specific, and highly sensitive determination of histidine. The method is based on the fluorescence derivatization of histidine with orthophthalaldehyde in a carrier stream of neutral buffer. A linear calibration curve for histidine was obtained over the range of 0.02-1000 pmol per injection (10 μL) with the relative standard deviation of 0.58%

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at 1.0 pmol (n = 10) and with the detection limit (S/N = 8) of 15 fmol. Since the fluorescence derivatization is selective for histidine and the interfering substances are limited to glutathione and histamine, the flow injection method was applicable to the rapid and specific assay of histidine in deproteinized human serum, urine, and the extract of stratum corneum where the relative amounts of the interferents to histidine are considerably low.

The present method can analyze forty samples per hour. Histidine values in the biological samples obtained by the present method correlated well with those determined by high-performance liquid chromatography. The present method is also applicable to biological samples containing large excess amounts of glutathione, by pretreatment of the samples with a thiol masking reagent such as N-ethylmaleimide.

INTRODUCTION

Numerous methods have been reported for the determination of histidine, which include a spectrophotometric method using diazo coupling reaction,(1,2) a fluorometric method using the condensation reaction with orthophthalaldehyde (OPA) in an alkaline medium,(3) and an enzymatic method based on the spectrophotometric measurement of urocanic acid formed from histidine by histidine ammonia-lyase.(4,5) These methods, however, suffer from some disadvantages such as low specificity or insufficient sensitivity. Therefore, histidine in biological samples has been measured principally by a high-performance liquid chromatography (HPLC) with pre- or postcolumn chemical derivatization selective for a primary amine.(6) Though the HPLC methods can also quantify other amino acids, they are time-consuming and inadequate for the assay of histidine in a large number of biological samples, because all amino acids have to be separated from each other.

OPA has been known to give a fluorescent adduct with most primary amines in the presence of a thiol compound,(7) but only with several biological substances, such as histidine,(3,8,9) histamine,(8,10-12) and glutathione(13,14) in the absence of a thiol compound in a neutral or weakly alkaline medium. Under the optimized conditions for the detection of histidine, the fluorescence reaction with OPA alone showed no significant fluorescence with other biological substances, except for histamine and glutathione. The relative fluorescence intensities of histamine and glutathione were 14.4 and 11.8% of that given by histidine on a molar base, respectively.(9) By using this fluorescence reaction for the post column derivatization, we have recently developed the simple and sensitive HPLC method for the specific assay of serum histidine.(9) The chromato-

graphic conditions of this HPLC method had only to separate histidine from glutathione, because many biological samples normally contain histamine markedly less than histidine,(15-18) especially, the level of histamine in human serum or plasma is 10,000-fold lower than that of histidine.(15,19-25)

In the course of study on application of this HPLC method, however, glutathione was scarcely detected in human serum, urine, and stratum corneum, because its relative amount to histidine is also considerably low in such samples. These findings suggested that the on-line fluorescence derivatization with OPA enables the specific assay of histidine in human serum, urine, and stratum corneum, even without chromatographic separation.

The present paper describes the application of the fluorescence derivatization with OPA to a flow injection analysis (FIA) system for the rapid and specific assay of histidine in such biological samples. In addition, the FIA method was shown to be applicable to the assay of histidine in human blood, which contains glutathione at a level comparable to that of histidine, by pretreatment of the sample with a thiol masking reagent.

EXPERIMENTAL

Chemicals

L-Histidine monohydrochloride, glutathione, OPA, and a creatinine assay kit (based on Jaffe reaction) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Sodium octanesulfonate, histamine dihydrochloride, and N-ethylmaleimide (NEM) were obtained from Tokyo Kasei Organic Chemicals (Tokyo, Japan). HPLC-grade methanol was 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, a carrier solution, and reagent solutions.

A stock standard solution of histidine (10 mM) was prepared in 0.01 M HCl and stored in a refrigerator. Working standard solutions of histidine were prepared by diluting the stock solution with 0.01 M HCl before use. OPA solution was freshly prepared by dissolving in methanol.

Equipment

HPLC and FIA systems consisted of an L-6000 pump (Hitachi, Tokyo, Japan) and an LC-9A pump (Shimadzu, Kyoto, Japan) for deliveries of an eluent (or a carrier solution) and OPA reagent, a DGU-12A degasser (Shimadzu), a

Rheodyne Model 7725i sample injector with a 500 μL sample loop (Rheodyne, Cotati, CA, USA), a CTO-10A column oven (Shimadzu), an F-1050 fluorescence detector equipped with a 12 μL square flow cell, and a D-2500 data processor (Hitachi).

For centrifugation of human blood and serum samples, a Model 1910 micro refrigerated centrifuge (Kubota, Tokyo, Japan) was used.

Conditions for HPLC and FIA Methods

HPLC separation was performed at 40°C with a Develosil ODS UG-3 column (30×4.6 mm i.d., 3 μm, Nomura Chemical, Seto, Japan) as an analytical column, which was protected by a guard-pak cartridge column (Develosil ODS UG-5, 10×4.0 mm i.d., 5 μm), and with a 1:19 (v/v) mixture of methanol and sodium phosphate buffer (35 mM, pH 6.2) containing 5.3 mM sodium octanesulfonate as an eluent. OPA reagent used for post column detection of histidine was a 15:1 (v/v) mixture of 50 mM sodium phosphate buffer (pH 8.0) and 50 mM OPA in methanol. Both the eluent and OPA reagent were filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA) before use.

The eluent was delivered to the column at a flow rate of 0.5 mL/min through a preheater tube (stainless-steel tube, 10 m×0.8 mm i.d.) and the injector. Ten microliters of the 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, 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.

FIA was performed at 40°C with the same system used for HPLC, except, that the columns were replaced by a resistor PTFE tube (10 m×0.25 mm i.d.). As a carrier solution and a reagent solution, a 19:1 (v/v) mixture of 50 mM sodium phosphate buffer (pH 7.0) and methanol and a 15:1 (v/v) mixture of 50 mM sodium phosphate buffer (pH 7.0) and 50 mM OPA in methanol were used, respectively, at a flow rate of 0.5 mL/min. Both the solutions were filtered through the 0.45 μm membrane filter before use. Other conditions were identical to those used for HPLC.

Sample Preparation

Human serum (stored at -80°C before use) was mixed with an equal volume of 6% (w/v) perchloric acid in a 1.5 mL Eppendorf polypropylene micro test tube and was vortexed several times. The mixture was centrifuged at 10,000 *g* for 10 min at 4°C, then the supernatant was diluted 10 fold with water and was filtered through the 0.45 μm membrane filter. A portion of the filtrate was further diluted 10 fold with the carrier solution for FIA analysis or with 0.01 M HCl for HPLC analysis.

Human urine (stored at -20°C before use) was mixed with an equal volume of 6% (w/v) perchloric acid and was filtered through the membrane filter. The filtrate was diluted 1000 fold with 0.01 M HCl for HPLC analysis or, successively diluted, 10 fold each, with 0.01 M HCl, water and the carrier solution, respectively, for FIA analysis.

Human stratum corneum samples were prepared as reported previously.(26) Briefly, the stratum corneums were stripped by applying a cellophane adhesive tape (20 × 18 mm wide; Nichiban, Tokyo, Japan) to the skin in the region of the back of the hand for 10 seconds. Histidine on the tape was extracted by immersing the tape in 1.5 mL of 0.1 M KOH in a 2.0 mL screw-capped polypropylene tube for 30 min with occasional vortexing. The extract was acidified to pH 2 by adding 0.5 mL of 0.38 M HCl, and then filtered through the membrane filter. The filtrate was diluted 10 fold with the carrier solution for FIA analysis or with 0.01 M HCl for HPLC analysis. The tape-stripping experiments were done with 10 young female students (range 20-22 years) as volunteers, after their informed consents were obtained.

Blood was drawn from one of the authors into a heparinized Venoject tube (Termo, Tokyo, Japan). For HPLC analysis, the heparinized blood (1.0 mL) was mixed with water (0.9 mL) and 60% (w/v) perchloric acid (0.1 mL), vortexed, and then centrifuged at 4°C and 10,000 *g* for 10 min. The supernatant (400 μL) was transferred to an Ultrafree-MC centrifugal filter unit (Durapore type, 0.22 μm) (Millipore, Bedford, MA) and centrifuged at 4°C and 10,000 *g* for 1 min. A portion (50 μL) of the filtrate was added with 0.2 M NaOH (50 μL) and 1.0 mM NEM (900 μL) to the carrier solution. After standing at room temperature for 15 min, the mixture was diluted 10 fold with 0.01 M HCl and injected into the HPLC. For FIA analysis, the heparinized blood (0.2 mL) was mixed with water (0.8 mL) and 1.0 M perchloric acid (1.0 mL), vortexed, and centrifuged at 4°C and 10,000 *g* for 10 min. The supernatant (400 μL) was filtered by centrifugation at 4°C and 10,000 *g* for 1 min using the Ultrafree-MC centrifugal filter unit. A portion (25 μL) of the filtrate was added with 0.1 mM NEM (975 μL) to the carrier solution, allowed to stand at room temperature for at least 15 min, and used as a blood sample. As an external standard for calibration, histidine solution was treated with NEM in the same manner.

RESULTS AND DISCUSSION

HPLC Chromatograms of Human Serum, Urine, and Stratum Corneum

As reported previously,(9) we developed a simple HPLC method for the assay of serum histidine, which consisted of (1) deproteinization of serum with 5-sulfosalicylic acid (5-SSA), (2) separation by the reversed-phase ion pair chromatography, and (3) the post column fluorescence detection with OPA under neutral pH. In this study, however, we used perchloric acid instead of 5-SSA as a precipitant for protein, because 5-SSA was coeluted with glutathione at 1.4 min and showed unnegligible fluorescence.

Figure 1A shows the typical chromatogram of deproteinized human serum obtained by our HPLC method. The high selectivity of the post column detection made the chromatogram quite simple, where the peak due to histidine appeared at

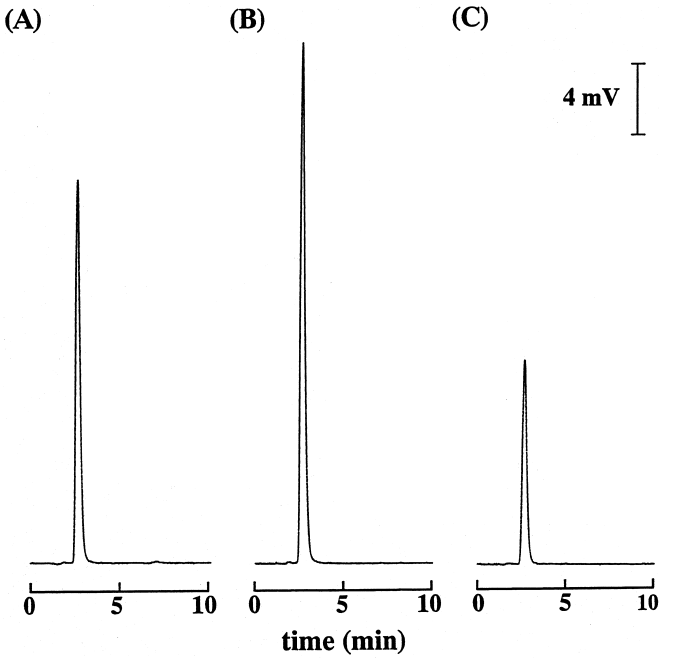

Figure 1. Typical HPLC chromatograms of deproteinized human serum(A), urine(B) and the extract of the stratum corneum from the back of the hand (C). Injected amounts of histidine were estimated to be 4.60, 6.25 and 2.45 pmol for serum, urine, and stratum corneum samples. The sample preparations and HPLC conditions were described in the Experimental.

2.7 min with no other peaks including that due to glutathione at 1.4 min. As shown in Fig. 1B, a similar chromatogram was also obtained with human urine sample. These results are reasonable because the content of glutathione is less than 5% of that of histidine in human plasma(27-29) and urine,(30) and because the relative fluorescence intensity of glutathione is 11.8% of that given by histidine on a molar basis in the fluorescence derivatization with OPA.(9)

Though no data are available for the level of glutathione in human stratum corneum, histidine is produced from the histidine-rich protein of the keratohyalin granule in the epidermis(31) and presents in the stratum corneum at a relatively high level.(26) Figure 1C shows the chromatogram of the extract of human stratum corneum from the skin of the back of the hand, where the peak due to histidine appeared as a sole peak. These results indicate that the fluorescence derivatization enables the specific assay of histidine in the deproteinized human serum, urine, and the extract of stratum corneum without the chromatographic separation.

The fluorescence derivatization was sensitive enough to detect histidine in serum and urine, even by a batch method. However, the fluorescent adduct of histidine was too unstable to be measured with satisfactory reproducibility by a batch method. In contrast, high reproducibility was attainable when the derivatization was applied to the on-line detection of histidine under precise controls of reaction time and temperature.(9)

FIA Method for the Assay of Histidine

Under the similar conditions optimized for the post column detection of histidine in our HPLC method,(9) the fluorescence derivatization with OPA in the present FIA method was performed for 30 seconds (reaction tube, 2.5 m×0.5 mm i.d.; total flow rate, 1.0 mL/min) at 40°C and at pH 7.0, by using a 19:1 (v/v) mixture of 50 mM sodium phosphate buffer (pH 7.0) and methanol as a carrier solution and a 15:1 (v/v) mixture of 50 mM sodium phosphate buffer (pH 7.0) and 50 mM OPA in methanol as a reagent solution. Figure 2A shows the FIA-grams of standard histidine recorded in a series of triplicate runs. The peak heights observed were reproducible and directly proportional to the injected amounts of histidine.

A linear calibration curve for histidine was obtained in the range of 0.02-1,000 pmol per injection (10 μL). The linear regression equation was calculated as $y = 4.47 x + 106$ ($r = 1.000$), where y, x, and r are FIA peak height, injected amount (fmol) of histidine and correlation factor, respectively. The detection limit of 15 fmol was obtained from a signal-to-noise ratio (S/N) of 8. The relative standard deviations ($n = 10$) were 0.58, 0.31, and 0.34 % at 1.0, 5.0, and 20 pmol, respectively.

For the application of this FIA method to the assay of human serum histidine, deproteinization was an essential step in the sample preparation, because the FIA peak height due to the serum sample increased by a factor of about 1.8 with-

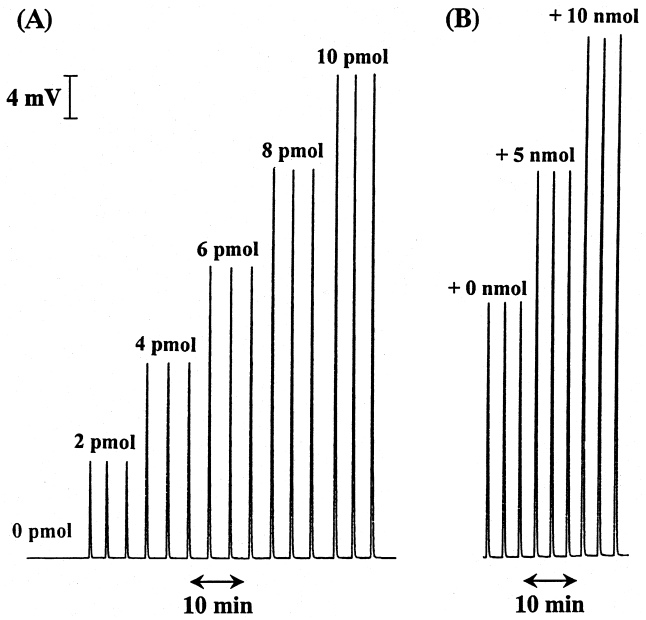

Figure 2. FIA grams of standard histidine (A) and serum samples to which were added with 0, 5, and 10 nmol/100 μL of standard histidine (B). The sample preparations and FIA conditions were described in the Experimental.

out deproteinization. Human serum albumin gave significant fluorescence in the derivatization with OPA. On a molar basis, its intensity was about 10% of that given by histidine and its serum level was 7-8 fold higher than that of histidine. Thus, serum albumin seemed to be mainly responsible for the difference in the FIA peak height between the serum samples with- and without deproteinization.

Figure 2B shows the FIA-grams of human serum to which were added standard histidine prior to the sample preparation. The amounts of histidine added to serum were 0, 5.0, and 10.0 nmol per 100 μL of serum, which corresponded to 0, 2.5, and 5.0 pmol per injection (10 μL), and gave the reproducible and proportional increases in the FIA peak height. Similar results were also obtained with histidine added to human urine and extract of stratum corneum (data not shown).

Evaluation of the Present FIA Method

Recovery of the present FIA method was tested in detail by using a control human serum or a pooled human urine, to which were added various amounts of histidine (2-10 nmol per 100 μLof serum or 20-100 nmol per 100 μL of urine)

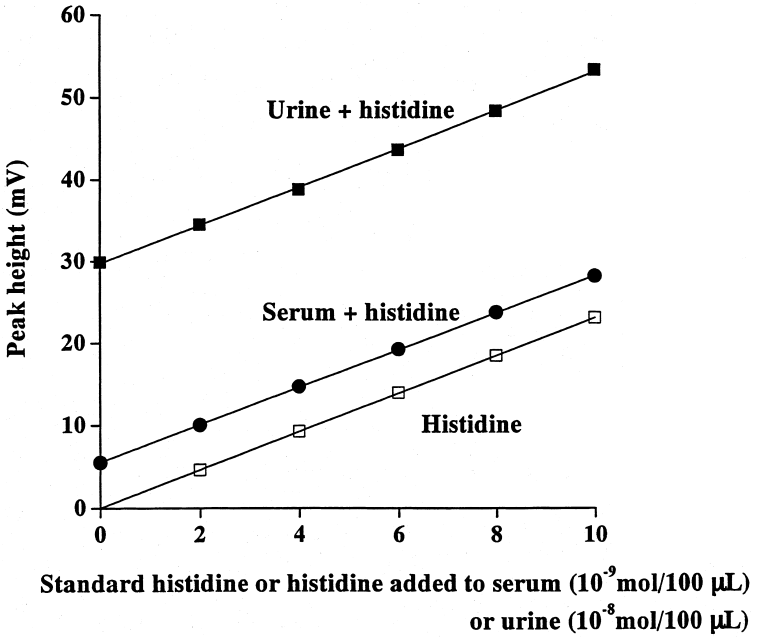

Figure 3. Peak height as a function of the amount of histidine in standard solution (□) and histidine added to human serum (2-10 nmol/100 µL, ●) and to urine (20-100 nmol/100 µL, ■).

prior to the sample preparation. As demonstrated in Fig. 3, the FIA peak height was a linear function of the amounts of histidine added to serum or urine with the slope closely similar to that obtained from standard histidine solutions. From the ratios of their slopes to that given by standard histidine solutions, the mean recoveries of histidine added to the serum and urine were estimated to be 103.8% and 101.2%, respectively. The relative standard deviations (n = 10) were 0.63 and 0.80% at 2 and 10 nmol per 100 µL of serum, and 0.61 and 0.98% at 20 and 100 nmol per 100 µL of urine, respectively (Fig. 3).

The correlation between histidine values obtained by the present FIA method and the HPLC method(9) was examined by assays of sera from healthy persons (n = 58). As shown in Fig. 4, there was a satisfactory linear correlation between the concentrations (µM) obtained by the two methods, and the linear regression equation was calculated as $y = 0.985\ x - 0.862$ ($r = 0.998$), where y is the value by the FIA method and x is the value by the HPLC method. The value of histidine in the same human sera (n = 58) determined by the FIA method was 86.4±14.4 µM (mean ± SD). The relative standard deviations of the present FIA method were also measured by using a pooled human serum to be 0.89% (n = 10) for the within-run and 0.96% (n = 7) for the day-to-day run.

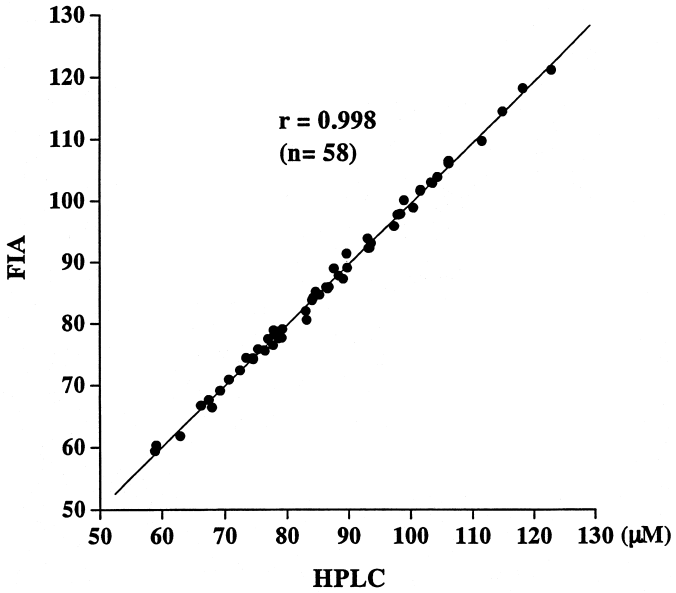

Figure 4. Correlation between histidine values in human sera (n = 58) determined by the present FIA method (y) and by the HPLC method (x). The linear regression equation is y = 0.985 x – 0.862 (correlation coefficient = 0.998).

In the same manner, histidine concentrations in human urine and stratum corneum of healthy volunteers (n = 10) were measured by the FIA method to be 1.13±0.47 μmol/mg creatinine and 481±382 pmol/cm^2 (on the skin of the back of the hand), respectively. These concentrations also correlated well with those obtained by the HPLC method. Namely, the linear regression equations were calculated as y = 0.977 x + 0.019 (r = 0.999) and y = 0.993 x + 5.0 (r = 0.999) for human urine and the stratum corneum, respectively, where y is the value by the FIA method and x is the value by the HPLC method.

The results described above, indicate that the present FIA method is applicable to the specific and precise determination of histidine in human serum, urine, and stratum corneum, in which the relative amount of glutathione to histidine is considerably low.

Application of the FIA Method to the Assay of Blood Histidine

The FIA method is not applicable directly to other biological samples such as liver, kidney, bile, erythrocytes, and whole blood, because they contain glu-

tathione at levels comparable to or higher than that of histidine.(30,32-34) However, the interference from glutathione seemed to be avoided by pretreatment of the samples with a thiol-masking reagent such as NEM, because the thiol group participates in the formation of the fluorescent adduct of glutathione with OPA.(34,35) This possibility was briefly examined by using human whole blood as a sample. Figure 5A shows HPLC chromatograms of a 1:100 mixture of standard histidine and glutathione with- and without NEM-pretreatment. The peak due to glutathione completely disappeared by NEM-pretreatment, whereas the peak due to histidine was unaffected. A similar result was also obtained with deproteinized blood as shown in Fig. 5B. Therefore, the present FIA method is also applicable to the specific assay of blood histidine in combination with NEM-pretreatment.

Figure 6A shows FIA-grams of a 1:100 mixture of histidine and glutathione, where NEM-pretreatment decreased FIA peak height to the value due to histidine alone. In combination with NEM-pretreatment, the present method gave a linear

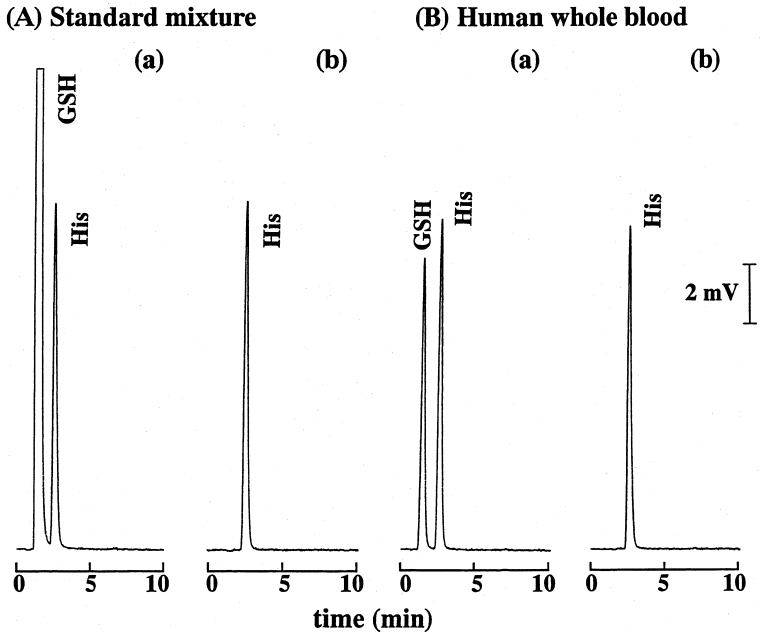

Figure 5. HPLC chromatograms of a mixture (100 μL) of standard histidine and glutathione (A) and deproteinized human blood (B) with (b) and without (a) pretreatment with N-ethylmaleimide (1.0 mM, 900 μL). The standard mixture contained 25 μM histidine (His) and 2.5 mM glutathione (GSH). The sample preparations and HPLC conditions were described in the Experimental section.

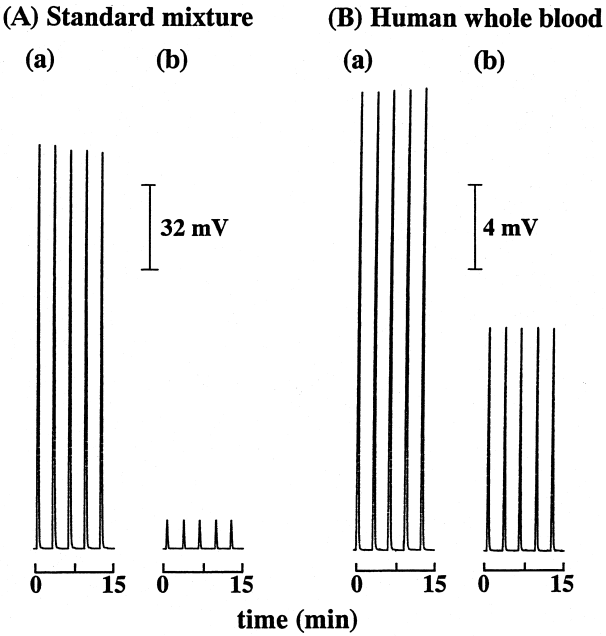

Figure 6. FIA-grams of a mixture (25 μL) of standard histidine and glutathione (A) and deproteinized human blood (B) with (b) and without (a) pretreatment with N-ethylmaleimide (0.1 mM, 975 μL). The standard mixture contained 10 μM histidine and 1.0 mM glutathione. The sample preparations and FIA conditions were described in the Experimental section.

calibration curve (r = 1.000) for 0.4-40 μM histidine solutions containing 1.0 mM glutathione, with the relative standard deviation of 0.57 % at 10 μM (n = 10). Figure 6B shows typical FIA-grams of deproteinized human blood with- and without NEM-pretreatment. Histidine value in the blood was estimated by this FIA method to be 97.0 μM with the relative standard deviation of 0.52 % (n = 10), which was in good agreement with the value (96.8 μM) obtained by HPLC method. These results indicate that the combination of the present FIA method with NEM-pretreatment enables the quantitative assay of histidine in blood and other biological samples containing a large excess amount of glutathione.

The present FIA method has several advantages over previously reported methods for the assay of histidine in terms of specificity, sensitivity, simplicity, and rapidity. The method is applicable to the specific assay of histidine in human serum, urine, and stratum corneum. Because of high sensitivity of the fluores-

cence detection with OPA, the present method permits quantitative determination of histidine in serum and urine, even diluted by a factor of about 400 and 4000, respectively. Furthermore, in combination with NEM-pretreatment, the present method is also applicable to other biological samples, which contain glutathione at levels comparable to or higher than that of histidine. Since the samples can be injected at 1.5 min intervals without overlap of the peaks, about 40 samples can be analyzed per hour.

The present FIA method is useful in the diagnosis of histidinaemia, one of hereditary metabolic disorders characterized by a virtual deficiency of histidine ammonia-lyase.(36) The enzyme is located mainly in liver and skin and catalyzes degradation of histidine to trans-urocanic acid.(37) The diagnosis of histidinaemia is, thus, made on the basis of the elevated histidine level in serum, urine, and the stratum corneum, for which the present FIA method seems to be well suited as a rapid and precise screening method for the assay of histidine.

ACKNOWLEDGMENTS

We thank the students of the College of Medical Technology of Hokkaido University for providing urine samples and for participating in the single tape-stripping experiments on the skin as volunteers. We also thank Sapporo Postal Life Insurance Examination Center for kindly providing serum samples.

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Received June 10, 2001 Manuscript 5596
Accepted July 6, 2001