



Development and validation of a highly sensitive LC–MS/MS method for *in vitro* measurement of histamine concentration

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ABSTRACT

The basophil histamine release test (HRT) is an important *in vitro* diagnostic assay to evaluate immunoglobulin E (IgE)-mediated allergic responses. In this study, a bioanalytical LC–MS/MS method was developed and validated to quantify histamine in the leukocyte suspension from human peripheral blood. The method used pre-column derivatization with phenylisothiocyanate (PITC) and the resulting phenylthiocarbamyl (PTC) histamine was analyzed by positive-ion electrospray ionization using the multiple reaction monitoring mode. Chromatographic separation was achieved using an Intakt-HT C18 column (2.1 mm × 50 mm, 3.0 μm), with a flow rate of 0.35 mL/min, 2 μL injection, and gradient elution with a mixture of acetonitrile–2 mM ammonium acetate buffer (both containing 0.1% formic acid). The total runtime of the method was 3.0 min including equilibration time. The method had a lower limit of detection of 0.1 ng/mL, and the quantifiable range was 0.1–100 ng/mL in the leukocyte suspension. The intra-day and inter-day precision and accuracy results were within the acceptable limits. It was established that histamine quantification should be performed within 2 h of preparing the leukocyte suspension, and freezing and thawing should be avoided. This method was successfully applied to the diagnosis and evaluation of the pathophysiologic mechanism of respiratory or cutaneous allergic diseases.

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1. Introduction

Histamine (HA) is a biogenic amine that plays a significant role in allergic response as a mediator that is released upon immunoglobulin E (IgE)-receptor activation of human mast cells and basophils [1]. The histamine release test (HRT) is an *in vitro* method to measure HA release from human basophils using either whole blood or leukocytes [2].

Currently, numerous detection methods have been described for measuring HA, including immunological assays such as radioimmunoassay (RIA) or enzyme-linked immunoassay (ELISA), fluorometric (automated or glass fiber based) technique, and chromatographic methods. RIA has been commonly considered as the gold standard for the *in vitro* quantitative analysis of HA because of their high sensitivity [3]. However, RIAs are time-consuming and produce radioactive wastes, which limit their application [4,5]. ELISA is commercially available and can be easily

performed according to the manufacturer's instructions. Furthermore, it only requires a small volume of the blood sample and no specialized equipment [6]. Nevertheless, the specificity of this assay remains a problem because the antibodies are often interrupted by 3-methylhistamine (the main metabolite of HA) or other low-molecular weight molecules. In addition, results may vary depending on the skill set of the analyst [7,8]. The fluorometric technique is perhaps the most common assay for determining the *in vitro* release of HA from basophil or mast cell cultures because a large number of samples can be processed with reproducibility and ease. However, this technique is less sensitive than immunoassays and requires large volumes of blood [9,10].

Chromatographic methods used to measure HA levels include capillary electrophoresis (CE), gas chromatography (GC), liquid chromatography (LC) with fluorescence, LC with electrochemical detection (ECD), and LC-tandem mass spectrometry (LC–MS/MS) [11–15]. Among these methods, LC–MS/MS plays a significant role in clinical laboratories with applications in endocrinology, toxicology, pharmacology, and therapeutic drug monitoring because of its excellent selectivity and appropriate sensitivity compared to those of immunoassays [16–18]. Recently, Chimalakonda and co-workers

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developed a hydrophilic interaction chromatography (HILIC)-based LC–MS/MS method to determine the chemical-induced HA release in established *in vivo* and *in vitro* models [15]. Koyama et al. have described an LC–MS/MS method to measure HA degranulation by KU812 cells [7]. Liu et al. also developed and validated an ultra-performance liquid chromatography (UPLC)–MS/MS method for the quantification of HA levels in plasma samples from various mammalian species [19].

The aim of the present work was to develop and validate a rapid, sensitive, and simple LC–MS/MS method for the determination of HA in leukocyte suspensions obtained from small sample volumes. In addition, the applicability of this method for the diagnosis of allergic diseases was also investigated by comparing it with the basophil activation test using flow cytometry.

2. Experimental

2.1. Materials and reagents

Histamine (CAS Number 51-45-6), internal standard (IS, Histamine- $\alpha,\alpha,\beta,\beta$ -d₄ dihydrochloride, CAS Number 344299-48-5), ACS reagent-grade formic acid, Triton™ X-100 detergent, 1,4-piperazine ethanesulfonic acid (PIPES), human serum albumin, calcium chloride, magnesium chloride, potassium chloride, sodium chloride, sodium hydroxide, dextran sulfate sodium, dextrose, ethylenediamine tetra-acetic acid (EDTA) solution, Tuerk solution, phenylisothiocyanate (PITC), and pyridine (>99.9%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile, methanol, and ethanol were obtained from Burdick and Jackson (Muskegon, MI, USA). Water was filtered using a Millipore Milli-Q system (Milford, MA, USA). Anti-human IgE antibody was purchased from KPL (Gaithersburg, MD, USA). *Dermatophagoides farinae* (*D. farinae*) extract was obtained from Prolagen Co. (Seoul, South Korea).

2.2. Stock solution preparation

Stock solutions of HA and IS were prepared in 50% methanol at concentrations of 1 mg/mL and stored at -20°C until dilution. Working solutions with different concentrations (1, 2, 5, 10, 50, 100, 500, and 1000 ng/mL of HA) were prepared by serial dilution with water. IS was diluted with water to prepare a stock solution at a final concentration of 200 ng/mL. The concentrations of quality control (QC) working solutions were 3, 400, and 800 ng/mL. All standard and quality control working solutions were kept at 4°C before analysis.

2.3. Preparation of standard and quality control samples

PIPES-ACMD buffer was used as a surrogate matrix for the leukocyte suspensions to prepare calibrators and QC samples. PIPES-ACMD buffer was prepared by adding albumin, calcium, magnesium, and dextrose to a 25 mM PIPES solution to give final concentration of 0.03% albumin, 0.1 M Ca^{2+} , 0.1 M Mg^{2+} , and 0.1% dextrose, respectively [20]. Calibration curves were prepared by spiking the HA working solution into the PIPES-ACMD buffer, and preparing serial dilutions that yielded eight calibration standards of 0.1, 0.2, 0.5, 1, 5, 10, 50, and 100 ng/mL. In addition, QC samples for HA were prepared in PIPES-ACMD buffer at concentrations of 0.3 ng/mL (QCL; low), 40 ng/mL (QCM; medium), and 80 ng/mL (QCH; high) in the same manner as the calibration curves.

2.4. Preparation of leukocyte suspension

Blood samples were obtained from patients with bronchial asthma and/or urticaria, who were sensitive to house dust mites

by positive specific IgE response to *D. farinae*, and four healthy volunteers. All subjects signed consent forms for samples to be used for research purposes. Leukocyte suspensions were prepared according to the dextran sedimentation procedure described previously with slight modifications [20]. Peripheral blood (2 mL) was collected in a tube containing EDTA. A mixture solution of 0.5 mL of 6% dextran-3% dextrose in 0.9% NaCl and 0.1 mL of 0.1 M EDTA was added to the whole blood sample to allow sedimentation of the red blood cells. The tubes were placed vertically for 60–90 min at 37°C . After sedimentation, the upper layer containing leukocyte-rich plasma was transferred to another tube, and centrifuged at 1400 rpm for 10 min at 4°C . After decantation and discarding the supernatant plasma, the leukocyte pellets were washed with 2 mL of PIPES-A (only including albumin) buffer and then resuspended with approximately 0.6 mL of PIPES-ACMD buffer. Aliquots (10 μL) were withdrawn for cell counting using a hemocytometer and residual leukocyte suspensions were prepared for measuring HA release.

2.5. Histamine release and derivatization

To evaluate HA release, 20 μL of the leukocyte suspension were added to 40 μL of different concentrations of HA-releasing stimuli (e.g., anti-IgE Ab) or allergens (e.g., *D. farinae*), and these mixtures were then incubated for 30 min at 37°C . Supernatants were obtained after centrifugation at 13,000 rpm for 10 min at 4°C . The HA derivatization reaction was performed using PITC reagent (Fig. 1). A 20 μL aliquot of the supernatant was transferred to a 1.5 mL micro tube and 10 μL of IS (200 ng/mL in water) was added. After the mixture was vortexed, 60 μL of PITC solution (PITC:ethanol:pyridine, 0.1:10:10) was added. The reaction was allowed to proceed for 10 min at 60°C and the samples were dried under N_2 at 40°C . The samples were reconstituted in 100 μL of the mobile phase. Finally, 2 μL of the sample was injected into the LC–MS/MS system.

2.6. Liquid chromatography and mass spectrometry

Chromatographic separation was performed on a Waters Liquid Chromatographic system (UPLC, Acquity I-Class, Milford, MA, USA) combined with a Waters Acquity binary solvent manager (BSM), an Acquity sample manager (SM), column heater, and degassing system. Chromatographic separation was performed on an Imtakt-HT C18 column (2.1 mm $\times$ 50 mm, 3.0 μm) maintained at 30°C . The mobile phase comprised 2 mM ammonium acetate with 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B). The flow rate was 0.35 mL/min with the following gradient conditions: 0–0.5 min, 10% B (isocratic); 0.5–1.5 min, 10%–80% B (linear gradient); 1.5–2.0 min, 80% B (isocratic); 2.0–2.1 min, 80% to 10% B (linear gradient); 2.1–3.0 min, 10% B (isocratic). Total run time was 3.0 min with 2 μL injection volume. Autosampler temperature was set at 10°C .

The analytes were detected by electrospray ionization mass spectrometry in positive ion mode on an API 4000 triple quadrupole mass spectrometer (MDS SCIEX, Toronto, Canada). Quantification was carried out using the multiple reaction monitoring (MRM) mode for the transitions m/z 247.1 $\rightarrow$ 154.1 and 251.1 $\rightarrow$ 158.1 for PTC-HA (phenylthiocarbamyl histamine) and PTC-IS (phenylthiocarbamyl internal standard), respectively. The source parameters of the mass spectrometer were optimized and maintained as follows: collision activated dissociation (CAD) gas, 4; curtain gas (CUR), 20; gas 1 (nebulizer gas), 60; gas 2 (heater gas), 60; turbo ion spray (IS) voltage, 5300 V; source temperature, 450°C . Nitrogen was used as the nebulizer, auxiliary, curtain, and collision

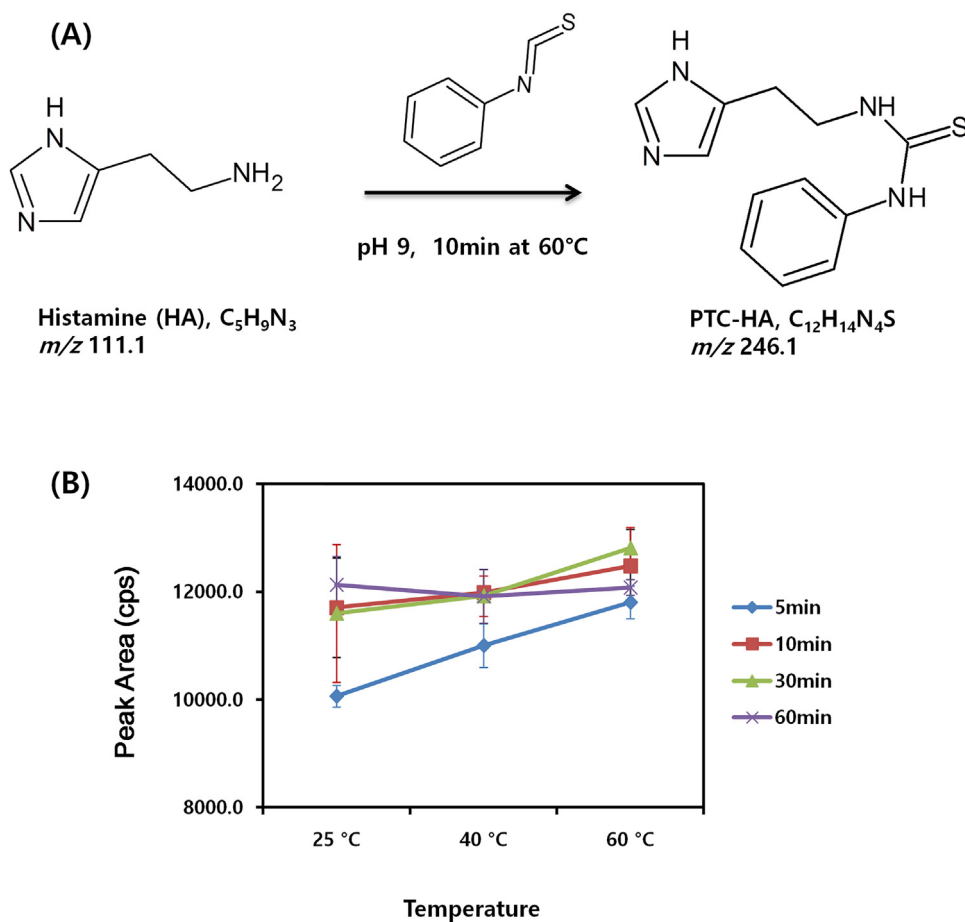


Fig. 1. (A) Derivatization reaction of histamine with phenylisothiocyanate (PTC) reagent. (B) Effect of time and temperature on the derivatization reaction of histamine ($n = 3$).

gases. Data processing was performed on Analyst 1.6.2 software (SCIEX).

2.7. Method validation

The method was validated for the determination of HA in PIPES-ACMD buffer as a surrogate matrix for leukocyte suspension for specificity, calibration, accuracy, precision, matrix effect, recovery, and stability according to the acceptance criteria of the FDA guideline for bioanalytical method validation.

2.7.1. Specificity

Specificity was evaluated by analyzing six different PIPES-ACMD buffer samples (different albumin concentrations ranging from 0.03% to 0.1%) to investigate the potential interference from co-eluting endogenous compounds in the LC peak region of PTC-HA and PTC-IS, respectively.

2.7.2. Linearity and lower limit of quantification

Calibration curves were constructed by plotting the peak area ratios (PTC-HA/PTC-IS) using $1/X$ weighted linear least-squares regression of the area ratio versus the nominal concentration of 0.1–100 ng/mL (0.1, 0.2, 0.5, 1, 5, 10, 50, and 100 ng/mL). The regression equation from the calibration standards was used to back-calculate the measured concentration for each standard. The lower limit of quantification (LLOQ) was the lowest concentration on the calibration curve that could be quantified with an acceptable precision of less than 20% and accuracy within $\pm 20\%$, which were evaluated using five replicate samples.

2.7.3. Precision and accuracy

The intra-day accuracy and precision of the assay were determined by analyzing three QC samples ($n = 5$) on the same day. The inter-day accuracy and precision were evaluated by the analysis of each QC sample over five consecutive days. Precision was determined on the basis of the coefficient of variation (C.V. (%)) and the accuracy was calculated as relative error (R.E. (%)). The acceptance criteria for precision and accuracy should be within $\pm 15\%$ compared to the nominal values.

2.7.4. Matrix effect and recovery

The matrix effect was evaluated ($n = 3$) by comparing the PTC-HA peak areas of the HA solution spiked with dried PIPE-ACMD buffer samples and the same neat solutions as the three QC samples. The recovery was determined ($n = 3$) by comparing the PTC-HA peak areas of three QC samples obtained in the PIPES-ACMD buffer and HA neat solution at these levels. The matrix effect and recovery of the PTC-IS were also evaluated using the same method.

2.7.5. Stability

HA stability was evaluated using three QC samples ($n = 3$) prepared in PIPES-ACMD buffer under various conditions. The short- and long-term stabilities were tested at room temperature (25 °C) for 6 h and -80 °C for 30 days, respectively. Freeze–thaw stability was evaluated after three freeze–thaw cycles (freezing at -80 °C; thawing at 25 °C) along with the freshly prepared QC samples. The QC samples were considered stable if the results were within 15% of R.E. (%) and C.V. (%).

In addition, the stability of basophils in leukocyte suspension was assessed with samples from four healthy volunteers by considering the amount of spontaneous HA release from various storage conditions.

2.7.6. Application

HA was measured in the leukocyte suspension by the described LC–MS/MS method. HA release was measured from both unstimulated and stimulated cells. Unstimulated cells incubated with only PIPE-ACMS buffer represent spontaneous histamine release (H0), whereas stimulated cells indicate histamine release (Hx) in response to a stimulator. Total histamine (H100) content was determined by lysing the cells with Triton™ X-100 detergent. Histamine release test (%) was calculated as follows: $[(Hx-H0)/(H100-H0)] \times 100$.

Basophil activation test (BAT) was performed with human whole blood using the Flow CAST™ kit (Buhlmann Laboratories AG, Schönenbuch, Switzerland) according to the manufacturer's instructions. Briefly, 50 μ L of EDTA-anticoagulated whole blood of the patient was incubated for 25 min at 37 °C with 50 μ L stimulator in the presence of 100 μ L stimulation buffer containing calcium and IL-3. Subsequently, staining reagents (Anti-CCR3-PE and anti-CD63-FITC) were added. After an incubation period of 15 min at 37 °C in a water bath, 2 mL of the pre-warmed lysing solution was added to the tube and incubated for 10 min at room temperature. After centrifugation and washing, the cells were suspended in 300 μ L wash buffer. The sample was analyzed with a BD FACSVerser™ flow cytometer (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA). The results of BAT were compared with those of HRT.

3. Results and discussion

3.1. Method development

A quantitative analysis of HA in leukocyte suspensions by LC–MS/MS was developed based on the following considerations. First, basophils are the source of many mediators such as histamine in blood leukocytes; therefore, they were used in *in vitro* assays to evaluate IgE-mediated responses. However, isolation of basophils from peripheral blood is a time-consuming and expensive approach. Therefore, leukocyte suspensions were used instead of isolating basophils. Second, it is almost impossible to obtain a histamine-free authentic matrix (leukocyte suspension) to prepare calibration standards. Therefore, a surrogate matrix approach was used among various alternative approaches, including the standard addition method, and surrogate analytes and surrogate matrix. Third, histamine is a polar compound with poor retention on a typical reversed-phase stationary phase. Therefore, a derivatization process was used to increase the sensitivity and retention of HA on a reverse phase HPLC mode. Finally, a pre-column derivatization with PITC combined LC–MS/MS method using PIPE-ACMD buffer as a calibration matrix was developed and validated for the determination of HA in leukocyte suspensions.

3.2. Optimization of derivatization

HA can be detected either directly or after derivatization on a reverse phase column. The direct detection method on a reversed-phase ion pairing or HILIC separation mode is generally rapid and simple. However, it is not appropriate for human biological fluids such as leukocyte suspensions with respect to sensitivity and selectivity. Therefore, it is limited to HRT application.

Several different derivatization methods have been reported for the determination of HA, such as pre- and post-column derivatization, followed by the detection of the derivatized HA using

LC-fluorescence and LC–MS/MS. Pre- and post-column derivatization are typically accomplished using reagents such as PITC, o-phthalaldehyde (OPA), dansylchloride, and 4-(1-pyrene) butyric acid *N*-hydroxysuccinimide ester [8,13,21,22].

PITC reacts readily with primary and secondary amines to form yellow colored products through nucleophilic aromatic substitution reaction at alkaline pH. It is volatile, making it possible to remove the excess reagent in vacuum, and thereby minimizing the possibility of reagent interference. Precolumn derivatization results in PTC derivatives that can be separated and quantified using reverse-phase HPLC column. Therefore, the pre-column PITC derivatization method was used in this study.

The amine group of HA and a phenyl group of PITC reacted easily in the presence of pyridine reagent to give a stable PTC-HA product, as shown in Fig. 1A. The optimal PITC solution concentrations (PITC:ethanol:pyridine, 0.1:10:10) of each reagent were chosen based on the previous study [23]. In addition, PTC-HA was derivatized very stably under PIPES-ACMD aqueous conditions, allowing for faster analysis by reducing the duration of sample preparation.

The efficiency of the reaction was investigated at different temperatures (25 °C, 40 °C, and 60 °C, Fig. 1B). The reaction progressed rapidly at the indicated temperature and the maximum peak intensity was reached in 10–30 min. In order to reduce the total analysis time and improve the throughput, the reaction was carried out at 60 °C for 10 min. PTC-HA was stable at 10 °C (autosampler temperature) for more than three days (data not shown).

3.3. Mass spectrometry and liquid chromatography

The *m/z* values of the precursor and product ions for PTC-HA and PTC-IS in positive ion ESI mode was investigated by direct infusion after derivatization. As a result, the single charged molecular ions $[M+H]^+$ of PTC-HA and PTC-IS were predominantly detected in the acidic aqueous mobile phase. The mass spectra for PTC-HA and PTC-IS showed protonated molecular ions $[M+H]^+$ peaks at *m/z* 247.1 and 251.1, respectively. The MS/MS parameters, including the declustering potential (DP), collision cell exit potential (CE), and collision energy (CE), were optimized to obtain the most stable and intense product ion for PTC-HA and PTC-IS. The most abundant and stable fragment ions were observed at *m/z* 154.1 and 158.1 for PTC-HA and PTC-IS, respectively (Fig. 2). Analytes were detected by tandem mass spectrometry using MRM of precursor-product ion transitions with 100 ms dwell time, at *m/z* 247.1 $\rightarrow$ 154.1 and *m/z* 251.1 $\rightarrow$ 158.1 for PTC-HA and PTC-IS, respectively.

The chromatographic conditions, including mobile phase compositions (ammonium acetate, ammonium formate buffer, acetonitrile, methanol, and various pH conditions) and various reverse phase analytical columns, were evaluated on the HPLC system in order to obtain good chromatographic separation for PTC-HA and PTC-IS. Finally, an Imtakt-HT C18 column (2.1 mm $\times$ 50 mm, 3.0 μ m) and a gradient mobile phase containing 2 mM ammonium acetate buffer with 0.1% formic acid and acetonitrile with 0.1% formic acid were selected as high intensity signals, appropriate peak shapes, and suitable retention times were observed for both PTC-HA and PTC-IS (Fig. 3).

3.4. Preparation of leukocyte suspension

Dextran sedimentation is often used to prepare leukocyte cells for HA release because it requires little technical expertise and is the least manipulative toward the cells [24].

First, 0.6 mL of the leukocyte suspension was obtained from 2 mL of whole blood using dextran sedimentation. As a result, it was uniformly distributed in the hemocytometer chamber; it was confirmed that there were about 500–600 basophils by the flow cytometer analysis (Fig. 4), and the levels of spontaneous HA release

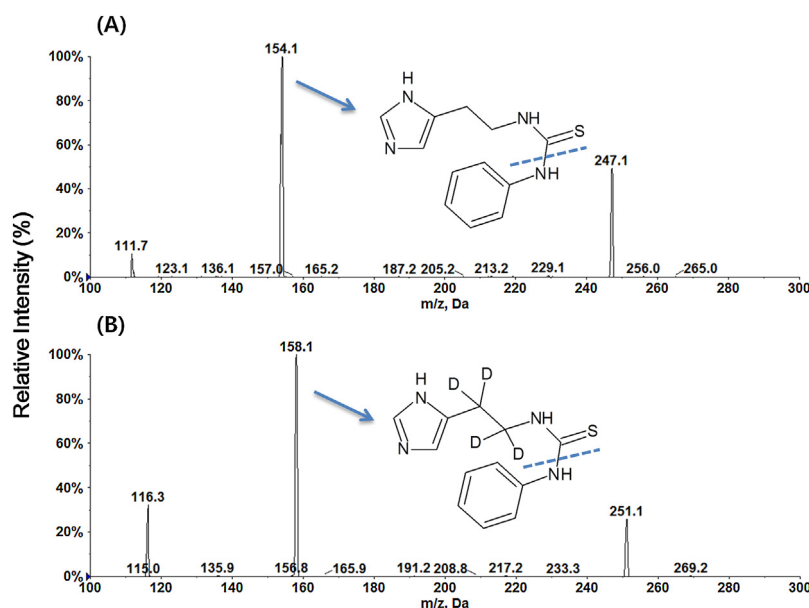


Fig. 2. Collision-induced dissociation mass spectrum of (A) PTC-HA and (B) PTC-IS in positive ion electrospray ionization mode. Product-ion spectrum from PTC-HA obtained by fragmentation of $[M+H]^+$ at m/z 247.1 and PTC-IS $[M+H]^+$ at m/z 251.1 using a collision potential of 21 eV.

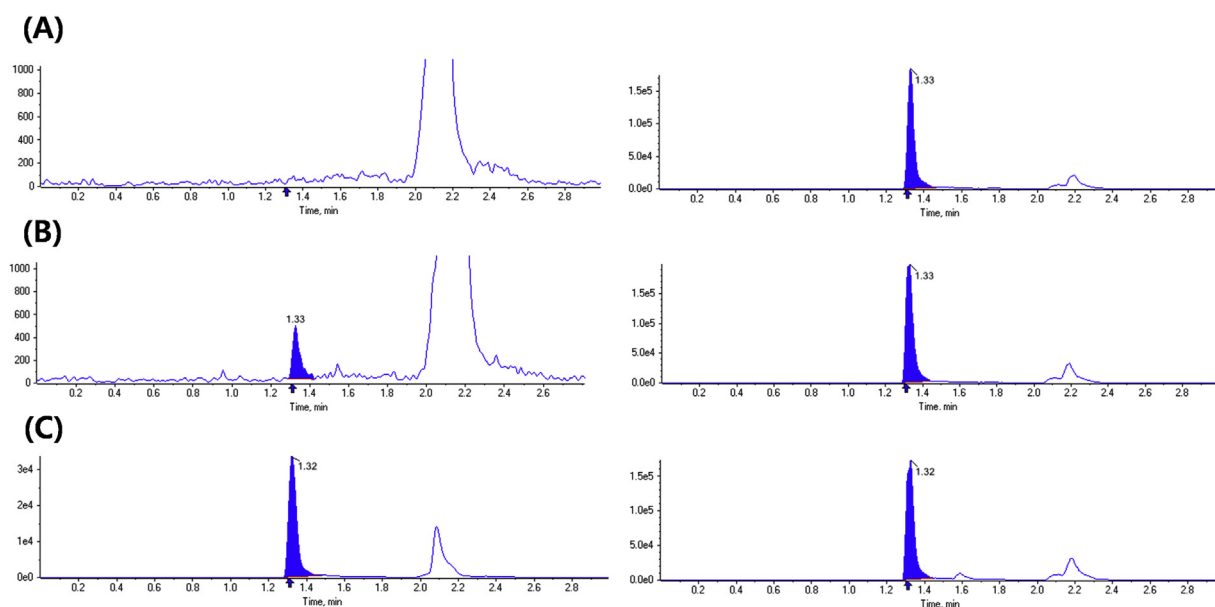


Fig. 3. Representative MRM chromatograms of (A) PTC-HA (left panel) and PTC-IS (right panel) in PIPES-ACMD buffer spiked with internal standard; (B) 0.1 ng/mL (LLOQ) histamine sample; (C) histamine release sample from leukocytes suspension treated with anti-human IgE antibody.

were found to be in the range of 0.5–28 ng/mL (data not shown). The results showed that this method can be used for multiple HRTs with only 2 mL of blood, and also suggested that relatively small amounts of the sample were sufficient for HRT in comparison to other studies, which typically require more than 10 mL of blood [20]. Therefore, this method is advantageous when repetitive blood sampling is required. As shown in Fig. 4, basophils in the leukocyte suspension exhibited similar activation patterns to basophils in whole blood. These results suggested that the basophils were not activated or manipulated during the preparation of the leukocyte suspension, which meant that this process is not problematic.

Secondly, LC–MS/MS requires sample clean-up to remove interferences (e.g., phospholipids and proteins) from its matrices before analysis. However, the leukocyte suspension does not require an additional sample clean up procedure because it has a lower protein

amount (below 800 μ g/mL) compared to that of the plasma sample. This had no effect on the efficiency of the PTC derivatization method. The results indicated that HA could be rapidly measured because no pre-treatment was required.

3.5. Method validation

3.5.1. Specificity

The number of leukocytes may vary for each sample depending on age, gender, individual differences, and blood volume. Therefore, the amount of protein in the leukocyte suspension obtained from several patients was measured; less than 400–800 μ g/mL of protein was detected. This indicated that the amount of albumin in the PIPES-ACMD buffer was about 0.03% to 0.1%. The specificity was measured with six PIPES-ACMD buffers having different amounts of

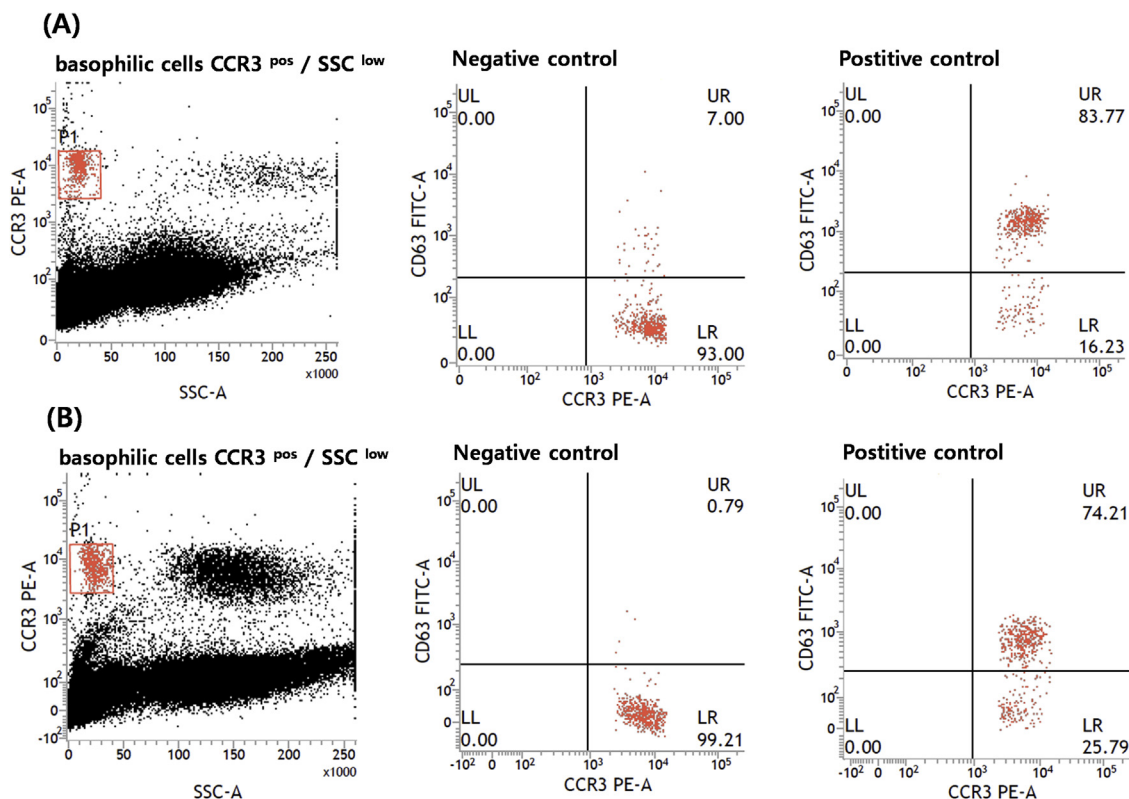


Fig. 4. Basophils were identified as CCR3^{pos}/SSC^{low} from the (A) leukocyte suspension and (B) whole blood of the same patient. In negative control, the samples were treated only with the stimulation buffer. In positive control, the samples were treated with anti-human IgE antibody.

Table 1
Linearity data for the analysis of histamine in PIPES-ACMD buffer (n = 5).

Nominal Concentration (ng/mL)	Calculated Concentration (ng/mL)	R.E. %	C.V. %	R ²
0.1	0.10	-2.00	13.30	0.99
0.2	0.19	-3.00	7.82	
0.5	0.54	7.20	4.87	
1	0.94	-6.20	7.25	
5	5.10	1.92	5.56	
10	10.30	2.96	4.17	
50	50.57	1.14	7.15	
100	99.07	-0.93	3.60	

R.E., relative error.

C.V., coefficient of variation.

R², coefficient of determination.

albumin. The representative MRM chromatograms of PTC-HA and PTC-IS are shown in Fig. 3. The retention times for PTC-HA and PTC-IS were all nearly 1.3 min. The peaks for their derivatized products were not interfered by the PIPES-ACMD buffer, which confirmed the specificity and selectivity of the method.

3.5.2. Linearity and lower limit of quantification

As shown in Table 1, the calibration curves showed good linearity over the concentration range, the observed deviation was within $\pm 15\%$ and the coefficient of determination (R^2) was above 0.99 for all calibration concentrations.

The LLOQ was defined as 0.1 ng/mL, which is the lowest concentration with an accuracy of $\pm 20\%$ and precision below 20%. Moreover, the values of R.E. and C.V. (n = 5) for LLOQ were -2.0% and 13.3%, respectively, demonstrating that this method is sensitive enough to determine the HA level in leukocyte suspensions.

Table 2

Precision and accuracy for the determination of histamine in PIPES-ACMD buffer (n = 5).

Nominal Concentration (ng/mL)	Intra-day		Inter-day	
	R.E. (%)	C.V. (%)	R.E. (%)	C.V. (%)
0.1	-1.80	9.59	4.40	11.90
0.3	5.33	6.17	2.80	6.46
40	2.70	8.31	3.80	6.25
80	-0.53	1.31	0.08	3.13

R.E., relative error.

C.V., coefficient of variation.

Table 3

Matrix effect and recovery of histamine in PIPES-ACMD buffer (n = 3).

Nominal Concentration (ng/mL)	Matrix effect (%)		Recovery (%)	
	Mean	C.V. (%)	Mean	C.V. (%)
0.3	99.87	6.27	102.49	5.81
40	95.30	3.79	98.98	1.95
80	101.37	2.78	101.50	0.54

C.V., coefficient of variation.

3.5.3. Precision and accuracy

As shown in Table 2, the intra-day precision and accuracy ranged from 1.31% to 9.59% and from -1.80% to 5.33%, respectively. The inter-day precision and accuracy ranged from 3.13% to 11.9% and from 0.08% to 4.4%, respectively. The results demonstrated that the values were within the acceptable range and the method was accurate and precise.

3.5.4. Matrix effect and recovery

Matrix effect and recovery were investigated at three QC levels, as described above, and the results are summarized in Table 3.

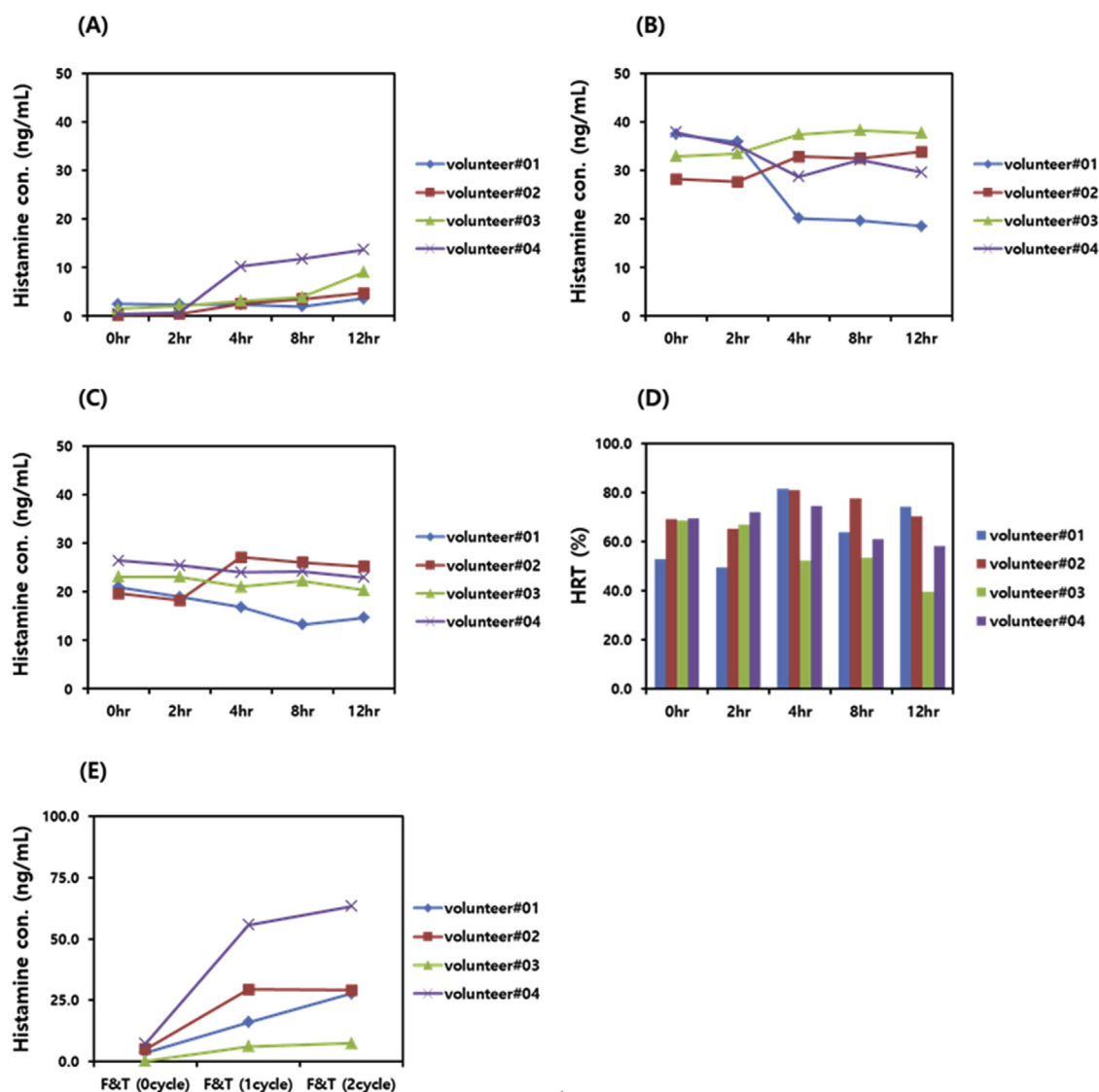


Fig. 5. Evaluation of the stability of leukocyte suspension by the amount of histamine release at the indicated condition. Samples were kept at 4 °C for the indicated time periods before use, and the amounts of histamine release were measured according to the indicated time. Samples were treated with (A) only buffer, (B) 0.1% Triton-X, and (C) anti-human IgE antibody (1 ug amount). (D) HRT (%) results for the indicated time. (E) Amount of spontaneous histamine releases from the freeze & thaw cycles.

These results indicate that the method provides satisfactory efficiency and no significant interference of co-eluting endogenous compounds with either PTC-HA or the PTC-IS.

3.5.5. Stability

The stability results showed that HA spiked in PIPES-ACMD buffer was stable for 6 h at room temperature, for 30 days at –80 °C, and during the three freeze-thaw cycles. The results of the stability experiments are listed in Table 4 and demonstrate that HA was stable under the described conditions.

The spontaneous HA release in the leukocyte suspension obtained from four healthy volunteers was found to increase slowly with time (Fig. 5A). The amount of histamine released by Triton™ X-100 detergent or anti-IgE antibody was observed to change rapidly after 2 h at 4 °C (Fig. 5B and C). As shown in Fig. 5D, a significant increase or decrease in HA release compared to the baseline result was observed after 2 h. In addition, the cell count of the leukocyte suspension decreased by less than 5% until 2 h, and thereafter decreased sharply up to 50% after 12 h. Therefore, the leukocyte suspension should be analyzed within at least 2 h at 4 °C.

The amount of spontaneous HA release increased significantly with the number of freeze-thaw cycles of the leukocyte suspension (Fig. 5E). It is thought that the spontaneous HA release increased as a result of breaking of the basophils during the thawing process. These results indicated that the leukocyte suspension could be kept in the refrigerator for up to 2 h and that it was better to avoid freeze-storage.

3.6. Application

Both HRT and BAT were simultaneously performed for patients with respiratory or cutaneous allergy in order to compare the results of these two assays. Leukocyte suspension and whole blood were utilized for HRT and BAT, respectively. As shown in Fig. 6, both experiments showed almost the same results, and HRT showed a dose-dependent pattern of HA release within the range of allergen concentrations. The same assays were performed for five patients altogether, and the results of the other four patients showed similar results (data not shown).

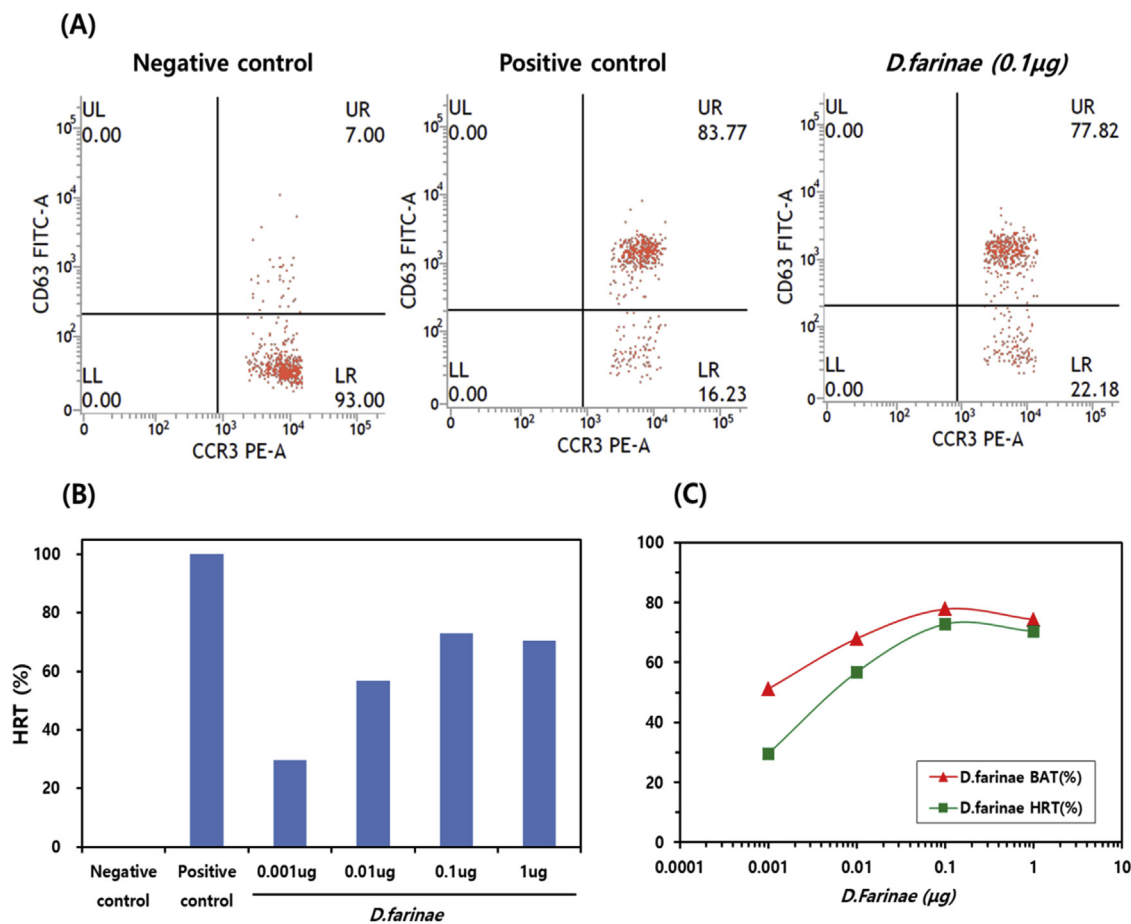


Fig. 6. Results of (A) the basophil activation test and (B) the histamine release test in the same patient with a respiratory allergy indicating sensitization to *D. farinae*. (C) Comparison of histamine release and basophil activation after stimulation with *D. farinae* in patient with house dust mite-sensitive asthma.

Table 4
Stability of histamine at three QC levels (n = 3).

Histamine	Concentration (ng/mL)		R.E. %	C.V. %
	Nominal	Calculated		
Short-term : exposure at RT for 6 h	0.3	0.33	9.28	9.36
	40	38.70	-3.24	3.85
	80	81.55	1.93	1.18
Long-term : storage at -80 °C for 30 days	0.3	0.30	1.31	8.28
	40	37.42	-6.45	0.64
	80	82.86	3.57	2.13
Freeze and thaw for 3 cycles : freezing at -80 °C and thawing at RT	0.3	0.31	2.96	9.68
	40	36.96	-7.59	0.89
	80	80.36	0.44	0.97
Post-preparation : auto-sampler (10 °C) for 12 h	0.3	0.30	-1.32	5.22
	40	37.53	-6.18	2.26
	80	81.67	2.09	1.07

RT, room temperature.

R.E., relative error.

C.V., coefficient of variation.

4. Conclusion

The described LC-MS/MS method is a sensitive, accurate, and robust assay for the determination of HA in leukocyte suspensions from human peripheral blood samples. This method involves a simple derivatization and requires only a small amount of blood (2 mL). In addition, preparation of an antigen dose-response curve requires only 0.1 mL of the leukocyte suspension and HA can be detected quickly (up to 20 samples every hour). This validated method

was successfully applied to patients with respiratory or cutaneous allergy. Thus, this method is suitable for routine or research use in medical laboratories.

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