

DRUG FORMULATIONS

Validation of HPLC Method for Determination of Histamine in Human Immunoglobulin Formulations

Michikazu Tanio ^{1,†}, Toru Nakamura,^{2,†} Hideki Kusunoki,¹
Kyohei Ideguchi,² Kazuyuki Nakashima^{2,*} and Isao Hamaguchi^{1,*}

¹Department of Safety Research on Blood and Biological Products, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo, 208-0011, Japan, ²KM biologics company, 1-6-1 Okubo, Kita-ku, Kumamoto-shi, Kumamoto, 860-8568, Japan

[†]These authors contributed equally to this work.

*Corresponding author's e-mail: nakashima-k@kmbiologics.com; 130hama@nih.go.jp

Abstract

Background: Histamine fixed-immunoglobulin formulations, which consisted of 0.15 µg of histamine dihydrochloride and 12 mg of human immunoglobulin in a vial, are used for anti-allergic treatments, and controlling the amounts of histamine in the formulations is essential to avoid histamine intoxication.

Objective: A high-performance liquid chromatography (HPLC) method for determination of histamine contents of the formulations was established and validated.

Methods: Histamine extracted from the formulation was labeled with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and was analyzed by gradient elution HPLC with UV detection at 260 nm.

Results: The method showed linearity in the range 0.8–2.4 µM ($R > 0.999$), accuracy (100.1–105.8% recovery), and precision (relative standard deviation $\leq 1.93\%$). The validated method was applied for five lots of the pharmaceutical, and their histamine contents were determined to be 0.149–0.155 µg/vial.

Conclusions: These results indicated that the validated method is useful to control amounts of histamine in biopharmaceutical products.

Highlights: The HPLC method was developed for quantitative determination of histamine content of the histamine fixed-immunoglobulin formulations.

Histamine, a biogenic amine, plays the main role in allergic responses (1–6). Histamine-fixed immunoglobulin formulations are used for anti-allergic treatments, because the formulation induces the development of specific antibodies to histamine (7, 8). Histaglobin® S.C. inj. (Nippon Zoki Pharmaceutical Co., Ltd., Japan), a histamine-fixed immunoglobulin formulation, consists of 12 mg of human immunoglobulin (Ig), 0.15 µg of histamine dihydrochloride (Hst) and 32 mg of sodium thiosulfate pentahydrate (Thi) as lyophilized powder, and has been used for treatment of allergic diseases such as bronchial asthma, allergic rhinitis, urticaria, and atopic dermatitis. Although the

formulation dissolved in 1.5 mL of water is subcutaneously injected into patients, excessive histamine in a body may cause histamine intoxication (2, 4, 9) and thus it is essential to control the histamine content in the formulation. As described in the notification from Ministry of Health, Labor and Welfare in Japan (Notification No. 0116-(1) of Pharmaceutical and Food Safety Bureau), the current identification test of histamine in the formulations has been performed by paper chromatography for spot-detection [retardation factor (R_f) = 0.57] and by fluorometric analysis for intensity-detection (≤ 0.5 µg Hst/vial), using o-phthalaldehyde (OPA) as the fluorescence reagent for

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histamine extracted from the formulation. However, these methods show low accuracy and specificity: the results with paper chromatography are often affected by the experimental conditions, and the detected fluorescence intensity can overlap with peaks arising from other OPA-labeled compounds that cannot be removed by extraction procedures. A quantitative method with high accuracy, precision, and specificity is therefore required for histamine detection.

Since the histamine molecule does not contain the specific and sensitive π conjugated systems for discrimination from other compounds by UV-visible detection, labeling of histamine is essential for its enhanced detection. Although several labeling reagents, such as OPA, fluorecamine, and 2,3-naphthalenedicarboxaldehyde, are available for histamine detection (7, 8, 10–14), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) is one of the most useful reagents for labeling of histamine as well as amino acids (15–18). The AQC-labeled compounds are stable for at least one week at room temperature, and therefore long-term analysis is possible (15). In addition, although AQC reagent is usually used as a fluorescent derivatizing reagent, AQC-labeled compounds can also be detected by UV at around 260 nm (15, 16), indicating that AQC-labeled compounds, such as the AQC-labeled histamine, can be analyzed with general HPLC systems without a fluorescence detector. In the present study, a quantitative HPLC method with UV detection using AQC as the labeling reagent was established and validated for determination of histamine extracted from the formulations. The validated method was used for assay of the histamine contents of five lots of Histaglobin.

Materials and Methods

Materials

Histamine dihydrochloride (Hst; $\geq 97.0\%$, $C_5H_9N_3 \cdot 2HCl$; FW, 184.07), L-histidine hydrochloride monohydrate (His), imidazole (Im), ammonium chloride (NH_4Cl), sodium thiosulfate pentahydrate (Thi; $Na_2S_2O_3 \cdot 5H_2O$), HPLC-grade trifluoroacetic acid (TFA), 1-butanol (for spectrochemical analysis), *n*-heptane (for spectrochemical analysis), hydrochloric acid (HCl; 35.0–37.0%), ammonium solution (28–30%), *o*-phthalaldehyde (OPA), phosphoric acid, sodium chloride (NaCl), boric acid, and sodium hydroxide (NaOH) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). HPLC-grade acetonitrile was obtained from Sigma-Aldrich Japan. High-purity water was supplied by a MilliQ system (Merck Millipore). Ig and two lots of Histaglobin (0278 and 0285) were provided by the KM biologics company. An AccQ-Tag™ Ultra Derivatization kit containing borate buffer, AQC reagent and acetonitrile reagent, and an ACQUITY UPLC® BECH C18 column (1.7 μm particle size, 2.1 mm $\times$ 50 mm) were purchased from Waters. For AQC-labeling without the Waters kit, AQC powder reagents ($\geq 90\%$ purity, Toronto Research Chemicals Inc.; TRC) were purchased from Cosmo Bio Co. Ltd., Japan. An NaCl-saturated 0.1 M NaOH solution was prepared by an addition of NaCl (35–40 g/100 mL) to 0.1 M NaOH. Three lots of Histaglobin (0350, 0359, and 0364) were purchased from the Nippon Zoki Pharmaceutical Co., Ltd.

Instrumentation

HPLC analysis was performed with an Agilent 1200 LC system equipped with a diode array detector, a binary pump, an autosampler with a thermostat, and a degasser. Data were collected and analyzed with Agilent ChemStation for LC 3D systems Rev.

B.04.03. Peak integrations were performed by drop-perpendicular method.

Standard and Sample Preparations

A stock solution of 1 mg/mL Hst (5.43 mM) was prepared by diluting 100 mg of Hst with 0.1 M HCl in a 100 mL-volumetric flask. Calibration standard Hst solutions (0.8, 1.2, 1.6, 2.0, and 2.4 μM) were prepared from the stock solution by dilution with water. 0.5 mL of each standard solution or 0.5 mL of water as a blank was transferred to a 15-mL tube.

The reconstituted Histaglobin sample was prepared by addition of 0.5 mL water to a vial of Histaglobin, and was then transferred to a 15-mL tube. The Hst concentration in the 0.5 mL-reconstituted Histaglobin is predicted to be 1.6 μM .

Glick-Extraction and AQC-Labeling

The formulation contains high concentrations of Ig (12 mg/vial) and Thi (32 mg/vial). The 0.5 mL-reconstituted formulation shows high viscosity due to the high concentration protein (24 mg/mL Ig). In the preliminary experiments, the high concentration of Thi in the reconstituted formulation (64 mg/mL Thi) was found to cause drastically decreased column performance and efficiency, resulting in broadening of peaks in the chromatograph. Deproteinization and desalting procedures are therefore essential for Hst detection by the HPLC method. A modified method of Glick et al (10, 13) was employed for Hst extraction, because the method is expected to remove proteins and salts from the sample during processes of organic solvent extractions.

About 0.3 g of NaCl and 0.5 mL of the NaCl-saturated 0.1 M NaOH solution were added to the 15-mL tube containing 0.5 mL of the sample solution. For the first extraction, 5 mL of 1-butanol was added to the 15-mL tube, and was shaken for 5 min to extract Hst into the 1-butanol. After centrifugation at $1920 \times g$ for 5 min, a 4-mL aliquot of the top butanol layer from the 15-mL tube was transferred to a 50-mL tube. For the second extraction, 4 mL of 1-butanol was added to the 15-mL tube, and shaken for 5 min. After centrifugation at $1920 \times g$ for 5 min, a 4-mL aliquot of the butanol layer from the 15-mL tube was collected to the 50-mL tube containing the first butanol extraction. 2 mL of 0.1 M HCl and 10 mL of *n*-heptane were added to the 50-mL tube. After shaking for 5 min, the tube was centrifuged at $1920 \times g$ for 5 min, and a 2-mL aliquot of the bottom aqueous layer containing Hst was transferred to a new 15-mL tube, which was then dried by rotary evaporation for 3 h to overnight at 32°C.

For AQC-labeling using the Waters kit, the dried sample was reconstituted with 80 μL of AccQ-Tag borate buffer, followed by addition of 20 μL of AccQ-Tag AQC reagent previously dissolved in 1.0 mL of AccQ-Tag acetonitrile reagent as according to the manufacturer's instructions. After heating at 55°C for 15 min, 300 μL of water was added to the reaction mixture, and the diluted solution was then filtered with a 0.22 μm filter unit (Millipore). The filtered solution was transferred to a 2 mL-sample vial for HPLC analysis.

As a robustness study, the reagents (AQC powder and borate buffer) used for AQC-labeling in the method were changed from those provided in Waters kit to other products; AQC powder from TRC and boric acid from Wako. About 10 mM AQC in HPLC-grade acetonitrile and 0.2 M borate buffer (pH 8.9) were prepared as labeling reagents in reference to the method by Cohen and Michaud (15). The calibration curve produced from the five Hst standards and the repeatability with the six replicates of the

1.6 μ M Hst standard using the TRC-produced AQC reagent indicated no statistically significant differences from those using the Waters kit with a significance level $\alpha=0.05$ (Supplemental Figure 1). In addition, it was confirmed that the peak area of the 2.4 μ M Hst standard labeled by AQC using the Waters kit or the TRC product showed almost no change for 8 or 7 days at 4°C, respectively (Supplemental Figure 2A), indicating AQC-labeled samples were stable for at least one week under the present experimental conditions, in good agreement with the previous report for AQC-labeled amino acids (15). Similar results were also obtained for AQC-labeled Hst extracted from Histaglobin using the Waters kit and the TRC-produced AQC reagent (Supplemental Figures 2B and 2C).

Chromatograph Conditions

The C18 column was kept on a column oven at 55°C and the autosampler thermostat was set at 4°C. Mobile phase solutions A and B were 0.1% TFA in water and 0.1% TFA in acetonitrile, respectively. The gradient conditions were 0.1% B for 1.08 min, 0.1–9.1% B for 10.4 min, 9.1–21.2% B for 4 min, 21.2–59.6% B for 0.6 min, 59.6–90.0% B for 0.02 min, 90.0% B for 1.18 min (rinse), and 90.0–0.1% B for 0.18 min, followed by re-equilibrium with 0.1% B for 7.54 min at a flow rate of 0.35 mL/min with 4 μ L of sample injection (total 25 min/run; Table 1). The elution peaks were monitored at 260 nm.

Validation Procedure

The method was validated according to the International Conference on Harmonization (ICH) guidelines for validation of assay procedures with respect to specificity, linearity, range, accuracy, and precision (October 28, 1997, Evaluating and Licensing Division of the Pharmaceutical and Medical Safety Bureau Notification No. 338; ICH Q2). All standard and sample preparations including the treatments of Glick-extraction and AQC-labeling were performed at each experimental day. The evaluation of linearity of the method was performed on four different days (Days 1–4), including three days (Days 2–4) to evaluate accuracy and precision as well as repeatability. The application of the method to five lots of the pharmaceutical was performed on other three different days (Days 5–7). An additional experiment was performed on one day (Day 8) to verify the identity between the calibration curves using TRC-produced AQC reagent and using Waters kit in the HPLC method. The

Table 1. Gradient conditions of the HPLC method. The C18 column was kept on a column oven at 55°C and the autosampler thermostat was set at 4°C. Mobile phase solutions A and B were 0.1% TFA in water and 0.1% TFA in acetonitrile, respectively. A flow rate is 0.35 mL/min and an injection volume is 4 μ L

min	Phase A (%)	Phase B (%)
0	99.9	0.1
1.08	99.9	0.1
11.48	90.9	9.1
15.48	78.8	21.2
16.08	40.4	59.6
16.10	10	90
17.28	10	90
17.46	99.9	0.1
25.00	99.9	0.1

summaries of the calibration curves from the eight days were shown in Supplemental Figure 3.

The means, standard deviations (SD), and confidence intervals of recoveries were calculated using AVERAGE, STDEV, and CONFIDENCE.T functions of Microsoft Excel for Mac 2011 (ver. 14.7.1), respectively. Relative standard deviations (RSD) in percentages were calculated as $100 \times [SD]/[mean]$. Confidence intervals of SD were calculated as square root of confidence intervals of population variances (σ^2) as the following inequalities using χ^2 distribution equation, $\frac{SD^2 \cdot (n-1)}{\chi^2_{\alpha/2, n-1}} \leq \sigma^2 \leq \frac{SD^2 \cdot (n-1)}{\chi^2_{1-\alpha/2, n-1}}$, where n is the sample size, and α is significance level. $\chi^2_{\alpha/2, n-1}$ and $\chi^2_{1-\alpha/2, n-1}$ are the values of χ^2 distribution with $n-1$ degree of freedom at probabilities of $\alpha/2$ and $1-\alpha/2$, respectively, as calculated using CHIINV function of Excel. Confidence intervals for RSD in percentages were calculated as $100 \times [confidence\ interval\ of\ SD]/[mean]$.

Specificity

1.6 μ M Hst, 1.6 μ M His, 1.6 μ M Im, 1.6 μ M NH₄Cl, 24 mg/mL Ig, 64 mg/mL Thi, and a mixture of these six compounds were prepared, and 0.5 mL of each sample as well as 0.5 mL of water as a blank was treated by Glick-extraction and AQC-labeling. The chromatograms of these samples were compared to evaluate specificity of the method.

Linearity and Range

The linearity of the method was evaluated on the basis of calibration curves obtained on four different days; the curves were constructed by plotting the absolute area of the Hst peak of each calibration standard treated by Glick-extraction and AQC-labeling as a function of Hst concentration (0.8, 1.2, 1.6, 2.0, and 2.4 μ M). Each regression line of the curves was calculated by the least-square method using Microsoft Excel for Mac 2011 (ver. 14.7.1). The evaluated range of the linearity was set between 0.8 and 2.4 μ M Hst, corresponding to 50% and 150% of the target concentration, respectively.

Accuracy and Precision

To evaluate the accuracy and precision, three samples were prepared with different Hst concentrations: one of the three samples contains 24 mg/mL Ig, 64 mg/mL Thi, and either 0.8, 1.6, or 2.4 μ M Hst. 0.5 mL of each sample was treated by Glick-extraction and AQC-labeling. The Hst concentrations of the three samples were calculated from the calibration curve on each of three different days. The percent recovery was estimated as the ratio of the calculated concentration to the theoretical one for each sample.

For additional evaluation of accuracy and precision, the Glick-extracted and AQC-labeled sample containing 1.6 μ M Hst, 24 mg/mL Ig, and 64 mg/mL Thi was measured with six replicates per day on three different days.

System Suitability Test

As the system suitability test for the HPLC method, the repeatability with six replicates of the absolute area and retention time of the Hst peak from the 1.6 μ M Hst standard treated with Glick-extraction and AQC-labeling were evaluated at each of the three experimental days for evaluation of the accuracy and precision (Days 2–4).

Paper Chromatography and Fluorometric Analysis

The current identification test of Hst in histamine-fixed immunoglobulin formulations for lot-release employs paper chromatography to confirm the existence of Hst ($> 0 \mu\text{g}/\text{vial}$) and fluorometric analysis to confirm the Hst content to be less than upper limit value ($\text{Hst} \leq 0.5 \mu\text{g}/\text{vial}$) in the pharmaceutical.

To estimate R_f values, the Glick-extracted Hst samples from the pharmaceutical (five vial/lot) and from a $0.75 \mu\text{g}$ -Hst standard solution as a control were analyzed by paper chromatography using the mobile phase containing 1-butanol, ammonia solution (28–30%), and ethanol with volume ratio of 8:3:1, respectively. The developed Hst on the paper was labeled by spraying with 0.2% OPA in methanol after spraying with 0.1M NaOH in methanol, and the spot of the OPA-labeled Hst was visually detected under UV light at 360 nm to determine the R_f value as migrated distance of the center of the spot divided by migrated distance of the solvent front.

For fluorometric analysis, each of the samples extracted by Glick-method from the pharmaceutical (one vial/lot) and from a $0.5 \mu\text{g}$ -Hst standard solution as a control was dried by rotary evaporation and was then reconstituted with 3 mL of water, followed by addition of 0.4 mL of 0.5M NaOH in methanol, and 0.15 mL of 0.1% OPA in methanol was then added to each of the reconstituted solutions for labeling reaction. After 30 min, 0.2 mL of 1.5M phosphoric acid in methanol was added to each solution to stop reaction. After more than 30 min, the fluorometric intensity of the OPA-labeled Hst in each sample was detected at 450 nm with excitation at 360 nm. The Hst content in a vial was calculated by intensity ratio of the pharmaceutical per the $0.5 \mu\text{g}$ -Hst standard.

Results and Discussion

Recovery by Glick-Extraction

The recovery of Hst by the Glick-extraction procedure was evaluated by comparing the peak area of the AQC-labeled Hst of the Glick-extracted standard sample with that of the corresponding standard sample untreated with Glick-extraction. The recovery of Hst by the Glick-extraction treatment was within 80.1–83.8%, and RSD between concentrations on each day or between days at each concentration was within 0.38–1.90% (Table 2). The mean recovery was 81.4% with an RSD of 1.05%, calculated on the basis of twenty determinations. These results indicated that the Glick-extraction procedure used in the method gave high reproducibility for a Hst concentration range of 0.8–2.4 μM .

Specificity

A typical HPLC chromatogram of Histaglobin using the method is shown in Figure 1A, and the focused chromatogram between the retention times at 6.5 min and 10.5 min is also shown in Figure 1B. The retention time of Hst was around 8.8 min under the present conditions. His, which is a precursor of Hst, Im and NH_4Cl , which are analogues of the decomposition products of Hst, and the pharmaceutical ingredients, Thi and Ig, were selected to investigate the specificity of the method. Each of the compounds or a mixture of all these compounds with Hst was treated by Glick-extraction and AQC-labeling, and their chromatograms were compared. No any peaks were observed between retention times of 8.0 and 10.5 min in chromatograms in the cases of His, Im, NH_4Cl , Thi, and Ig (Figure 2A). In contrast, a single Hst peak was observed at a retention time of around 8.8 min in the case of the mixed sample (Figure 2B), and the peak

Table 2. Recovery (%) of Hst by Glick-extraction ($n = 1$). The peak area of each Hst standard with Glick-treatment was compared with that of the corresponding standard without Glick-treatment

Hst	Day 1	Day 2	Day 3	Day 4	Mean	SD	RSD
0.8 μM	83.8	81.6	80.1	81.1	81.7	1.56	1.90
1.2 μM	80.2	81.4	81.0	81.4	81.0	0.58	0.72
1.6 μM	82.4	81.7	81.3	81.0	81.6	0.63	0.77
2.0 μM	81.0	82.5	82.1	80.6	81.5	0.90	1.10
2.4 μM	82.0	81.3	80.9	80.9	81.2	0.52	0.64
Mean	81.9	81.7	81.1	81.0			
SD	1.38	0.48	0.69	0.30			
RSD	1.68	0.58	0.85	0.38			
Total	Mean=	81.4	SD=	0.86	RSD=	1.05	

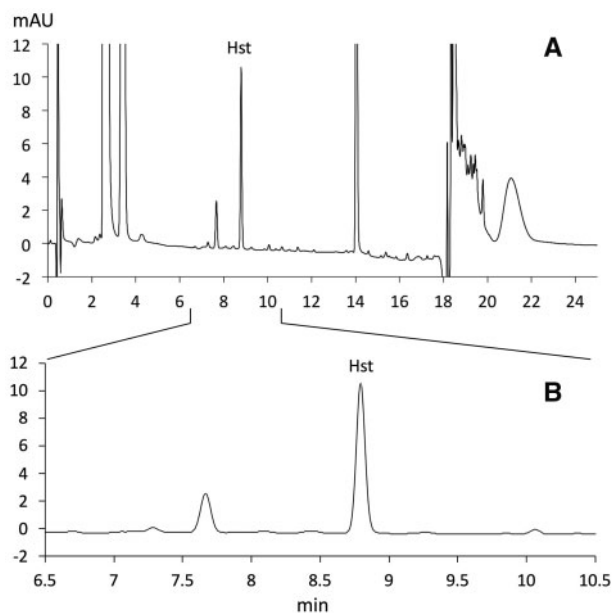


Figure 1. HPLC chromatogram of AQC-labeled histamine extracted from Histaglobin using the method (A). The labeled histamine was eluted at about 8.8 min of the retention time under the present conditions. (B) The chromatogram focused on the retention times between 6.5 and 10.5 min from (A). The peak area of Hst in the chromatogram was 48.814 mAU·s.

intensity and retention time of Hst were essentially identical to those of the $1.6 \mu\text{M}$ Hst sample (data not shown) and of the Histaglobin sample (Figure 1B). These results show that the HPLC method selectively detects Hst as distinguished from compounds similar or related to Hst, and from the pharmaceutical constituents, and that the observed single peak at around 8.8 min is ascribed to Hst only in Histaglobin as analyzed by the method.

The HPLC method was also applied to the AQC-labeled His, Im and NH_4Cl samples without the Glick-extraction procedure, and none of the chromatograms showed peaks at retention times between 8.0 and 10.5 min. The His peak was observed at a retention time of around 7.0 min, which is sufficiently separated from the Hst peak under the present conditions (Supplemental Figure 4A). The unidentified peak at around 7.6 min observed in all Glick-treated samples including the blank sample (Figure 2) disappeared in chromatograms of samples without Glick-extraction (Supplemental Figure 4A), suggesting that the

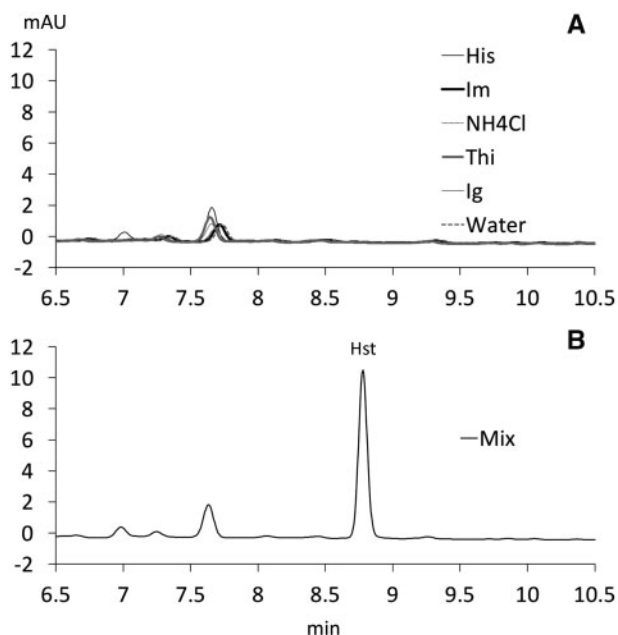


Figure 2. Specificity of the method. (A) Overlaying HPLC chromatograms of His, Im, NH₄Cl, Thi, Ig, and water (blank). No any peaks were observed at around 8.8 min in these chromatograms. (B) HPLC chromatogram of mixture of Hst, His, Im, NH₄Cl, Thi, and Ig. Samples were treated by Glick-extraction and AQC-labeling. The Hst peak was observed at about 8.8 min in the chromatogram with a peak area of 50.967 mAU·s. Chromatograms were focused on the retention times between 6.5 and 10.5 min.

unidentified peak may arise from contaminants during the Glick-extraction procedure.

Retention times (RT) of Hst peaks of 197 measurements from all samples and standards (with and without Glick-extraction) as obtained in the present study (eight experimental days) were summarized in [Supplemental Figure 4B](#). The mean of RT was 8.826 min with SD of 0.068 min ($n = 197$), corresponding to RSD of 0.8%, and all Hst peaks were observed within 8.7–9.0 min, in which the range has no any peak from compounds similar to or related to Hst and from the pharmaceutical constituents.

Linearity and Range

Each of the calibration curves obtained for the Glick-extracted and AQC-labeled standards on four different days ($n = 1$ per day) showed linearity for the Hst concentration range 0.8–2.4 μM ([Figure 3](#)). Linear regression analysis of these curves indicated that the mean $\pm$ SD values of the slope, y-intercept, and correlation coefficient (R) were 32.400 ± 0.308 , -0.816 ± 0.566 , and 0.9998 ± 0.0001 , respectively ([Table 3](#)). The four calibration curves overlapped almost perfectly, each of 95% confidence intervals (CI) of y-intercept of these curves included zero or near zero, and all the R values were greater than 0.999, indicating that the method has appropriate linearity and inter-day precision.

Accuracy and Precision

The percent recoveries of Hst from the three Hst-concentration samples on three different days ($n = 1$ per day) are summarized in [Table 4](#). At each Hst-concentration level, the recovery was within $\pm 10\%$ of the acceptable true value, and RSD for intermediate precision was less than 2.0%. The mean recovery from the nine determinations was 101.6% with RSD of 1.78%, and a 95%

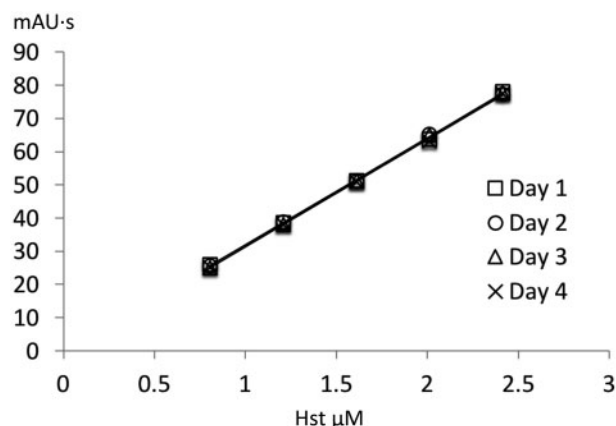


Figure 3. Linearity of the method. Four calibration curves produced on four different experimental days were overlaid. Concentration range is 0.8–2.4 μM of Hst, covering 50–150% of the target concentration.

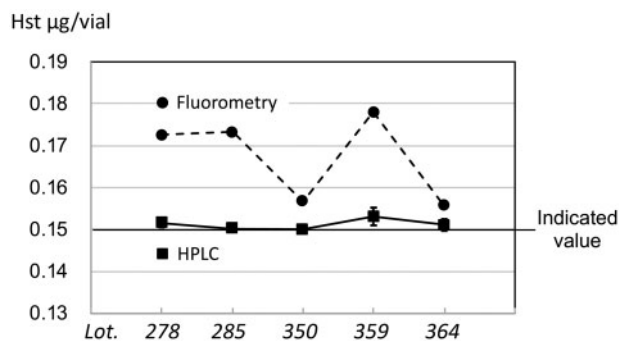


Figure 4. Hst contents of five lots of Histaglobin determined by the validated HPLC method (squares) were compared with those determined by the fluorometric analysis (circles) as employed by the current lot-release test. The HPLC data showed the average values from three days with bars of standard deviation for each lot.

confidence interval for recovery was 100.2–103.0%, indicating high accuracy and precision. These results also indicate that the presence of Thi and Ig hardly interferes with Hst detection by the method. Since calibration curves obtained from the three Hst-concentration samples with Thi and Ig were essentially identical with those obtained from the five Hst standards at each experimental day ([Supplemental Figure 5](#)), also indicating Thi and Ig have almost no influence on Hst detection by the method.

Repeatability ($n = 6$ per day) and intermediate precision (between three days) were also evaluated for the 1.6 μM Hst sample, corresponding to the 100% target concentration ([Table 5](#)). The mean recovery and RSD on each day were within 100.8–101.0% and 0.38–0.63%, respectively. One-way analysis of variance for the data in [Table 5](#) showed no significant differences between the three days ($F < 1$), and therefore it was concluded that the intermediate precision was essentially identical to the repeatability. A 95% confidence interval for RSD was 0.37–0.75% as calculated from the 18 determinations.

From these results, it was concluded that the method has appropriate accuracy and precision.

System Suitability Test

The repeatability with six injections using the 1.6 μM Hst standard showed RSD $\leq 1\%$ for both the absolute peak area and

Table 3. Linearity of the calibration curves obtained for the Glick-extracted and AQC-labeled standards in Figure 3

	Day 1	Day 2	Day 3	Day 4	Mean	SD
Slope	32.180	32.296	32.856	32.270	32.400	0.308
Intercept	-0.336	-0.408	-1.551	-0.969	-0.816	0.566
95% CI of Intercept	-2.997 ~ +2.325	-2.704 ~ +1.888	-2.705 ~ -0.397	-3.312 ~ +1.374	-	-
R	0.9997	0.9997	0.9999	0.9997	0.9998	0.0001

Table 4. Evaluation of accuracy and precision of HPLC method (n = 1) for three Hst concentrations. The three different Hst concentration samples containing Ig and Thi were analyzed by the method at each day

Hst	Recovery (%)					RSD
	Day 2	Day 3	Day 4	Mean	SD	
0.8 μ M	102.9	102.0	105.8	103.6	2.00	1.93
1.6 μ M	101.2	100.8	100.3	100.7	0.46	0.46
2.4 μ M	100.9	100.1	100.6	100.5	0.42	0.42
Total				101.6	1.81	1.78
95% confidence interval of recovery (%) : 100.2–103.0%						
95% confidence interval of RSD: 1.20–3.42%						

Table 5. Evaluation of accuracy and precision of HPLC method (n = 6) for 1.6 μ M Hst

Recovery (%)	Day 2	Day 3	Day 4	Total
Mean	100.8	101.0	100.8	100.8
SD	0.38	0.63	0.52	0.50
RSD	0.38	0.63	0.51	0.50
95% confidence interval of recovery (%) : 100.6–101.1%				
95% confidence interval of RSD: 0.37–0.75%				

The 1.6 μ M Hst sample containing 24 mg/mL Ig and 64 mg/mL Thi was analyzed by the method at each day.

retention time at each day (Table 6). In addition, RSD of retention times of the measured Hst peaks from all Hst-containing samples at the eight experimental days was also $RSD \leq 1\%$ (Supplemental Figure 4B). From these results, one candidate of the acceptance limit of RSD may be 3% for both peak area and retention time, corresponding to the three times of the maximum RSD observed (1%). Under the candidate limit of RSD, the concentration and retention time of 1.6 μ M Hst sample eluted at 8.8 min will be acceptable within 1.55–1.65 μ M and 8.5–9.1 min, respectively.

As an additional information, the results of the repeatability with six replicates of Column efficiency, Height equivalent to theoretical plates, Selectivity factor, USP Tailing factor, and USP Resolution of the Hst peak from the 1.6 μ M Hst standard were summarized in Supplemental Table 1. The mean values of the parameters met generally desirable criteria as referring to FDA CDER Reviewer Guidance Validation of Chromatographic Methods (1994) and USP General Chapter <621> Chromatography: Column efficiency >2000, Selectivity >1, USP Tailing ≤ 2 , and USP Resolution >2.

Application of the HPLC Method to the Pharmaceutical

The Hst contents of five lots of Histaglobin were determined by the validated method on three different days (n = 1 per day; Table 7). The Hst contents were within 0.149–0.155 μ g/vial, which are in good agreement with the indicated value for the pharmaceutical (0.15 μ g/vial).

Table 6. The repeatability with six replicates of the retention time and area of the Hst peak from the 1.6 μ M standard sample treated with Glick-extraction and AQC-labeling were evaluated at each experimental day for evaluation of accuracy and precision of the method (Days 2–4)

	Day 2	Day 3	Day 4
Absolute peak area (n = 6/day) mAU-s			
mean	51.174	51.322	51.135
SD	0.199	0.352	0.315
RSD	0.39%	0.69%	0.62%
Retention time (n = 6/day) min			
mean	8.855	8.848	8.833
SD	0.011	0.012	0.010
RSD	0.12%	0.14%	0.11%

Table 7. Hst contents (μ g/vial) of five lots of Histaglobin determined by validated HPLC method (n = 1)

Lot	Validated HPLC method (μ g/vial)						Identification test ^a	
	Day 5	Day 6	Day 7	Mean	SD	RSD	R _f value ^b	μ g/vial ^c
0278	0.151	0.153	0.151	0.152	0.001	0.67%	0.58 (0.60)	0.172
0285	0.150	0.151	0.150	0.150	0.000	0.28%	0.58 (0.58)	0.173
0350	0.149	0.150	0.151	0.150	0.001	0.42%	0.59 (0.58)	0.157
0359	0.155	0.154	0.151	0.153	0.002	1.38%	0.59 (0.60)	0.178
0364	0.151	0.152	0.150	0.151	0.001	0.94%	0.60 (0.59)	0.156
Mean	0.151	0.152	0.150	0.151 ^d			0.59 (0.59)	0.167
SD	0.002	0.002	0.001	0.002 ^d			0.01 (0.01)	0.010
RSD	1.34%	1.06%	0.50%	1.04% ^d			1.7 (1.4) %	6.17%
p ^e	0.015	0.020	0.020					

The indicated value for the pharmaceutical is 0.15 μ g Hst per a vial

^aThe criteria of the identification tests for lot-release are $R_f = 0.57$ by paper chromatography and Hst ≤ 0.5 μ g/vial by fluorometry.

^bR_f values for Hst standards under the same experimental conditions were shown in parentheses.

^cHst contents were estimated by fluorometric intensities for the pharmaceuticals as compared with those for the 0.5 μ g-Hst standards under the same experimental conditions.

^dThe mean, SD, and RSD were calculated pooling the total 15 determinations by the HPLC method.

^ep values were calculated by paired samples t-test for the five Hst contents on each lot between the HPLC method at each day and the fluorometric method.

Results of the same five lots by the current identification method for lot-release (paper chromatography and fluorometry) were also shown in Table 7. The R_f values of the pharmaceuticals were close to 0.57, and were essentially identical with those of the standard Hst samples under the same experimental conditions, identifying Hst in each pharmaceutical. Although the R_f values (0.58–0.60) were slightly larger than the criterion value of 0.57 for lot-release of the pharmaceuticals, R_f values are considerably affected by experimental conditions, which it is difficult to control strictly. Therefore, the identification of Hst was

practically assessed by comparing the R_f value for the pharmaceutical with that of the Hst standard under the same experimental conditions.

Although the Hst contents calculated by fluorometry met the criterion of $Hst \leq 0.5 \mu\text{g}/\text{vial}$ for lot-release, the estimated values ($0.156\text{--}0.178 \mu\text{g}/\text{vial}$) were slightly larger than the indicated value ($0.15 \mu\text{g}/\text{vial}$) with high variation of $RSD = 6.17\%$ calculated from the five lots (Figure 4 and Table 7). A paired samples t-test for Hst contents of the five lots on each lot between the HPLC method at each day and the fluorometric method indicated significant differences between both methods with a significance level of $\alpha=0.05$ (Table 7).

These results indicate that the current identification method showed lower specificity, accuracy and precision than the validated HPLC method.

Conclusions

The HPLC method was established and validated for the quantitative assay of histamine extracted by the modified Glick method from histamine-fixed immunoglobulin formulations using AQC labeling reagents. The validated method showed high accuracy, precision, and specificity, and is therefore useful for controlling the amounts of histamine in formulations. Although the HPLC method in the study was especially established for histamine-fixed immunoglobulin formulations, it is expected that the method can be applied to other biological products as well as foods under the same or slightly modified experimental conditions.

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Conflict of Interest

The authors declare no conflicts of interest associated with this manuscript.

Supplemental Information

Supplemental information is available on the J. AOAC Int. website.

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