A Modified Method for Measuring $N^\gamma$-Methylhistidine in Chiken Feed and Excreta

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Analysis of $N^\gamma$-methylhistidine is intricate and time consuming. In the present study, a modified technique using high-performance liquid chromatography, which utilizes a reversed-phase separation with ion pairing and post column fluorescence derivatization for the analysis of $N^\gamma$-methylhistidine in chicken feed and excreta is described. Two ml of the hydrolyzed sample was applied directly into the ion-exchange resin column without evaporation of HCl to roughly separate $N^\gamma$-methylhistidine from acidic and neutral amino acids. The $N^\gamma$-methylhistidine fraction was eluted with 1 M pyridine and then evaporated, and the residue was dissolved in the mobile phase and subjected to high-performance liquid chromatography. The present method shortened the time needed to complete the assay by about 30 min per sample and improved the separation of $N^\gamma$-methylhistidine.

Key words: $N^\gamma$-methylhistidine, high-performance liquid chromatography

Introduction

Protein metabolism is one of the dynamic processes inside the animal body. Growth is the result of protein accumulation and it is controlled by the difference between the rates of protein synthesis and breakdown (Hayashi et al., 1985, 1994; Yoshizawa et al., 1997). Muscles mainly consist of actin and myosin, and these proteins contain $N^\gamma$-methylhistidine ($N^\gamma$-MH) as their component. It is formed by the modification of histidine residues after the polypeptide chain is formed. $N^\gamma$-MH is neither broken down nor reutilized for protein synthesis because of the lack of the specific transfer RNA for this amino acid (Long et al., 1975; Young et al., 1972).

Many studies on small animals (David et al., 1996, 1998; Young et al., 1972), farm animals (Hayashi et al., 1987; Nagasawa et al., 1991) and humans (Long et al., 1975) have suggested that urinary $N^\gamma$-MH serves as a good index of skeletal muscle protein breakdown. In the case of chickens, $N^\gamma$-MH in feed and excreta (mixed feces and urine) has to be measured in order to calculate urinary $N^\gamma$-MH excretion. High performance liquid chromatography (HPLC) is usually used to measure $N^\gamma$-MH (David et al., 1996; Hayashi et al., 1987). However, as the HPLC methods
do not usually give reliable results when feed and chicken excreta are analyzed due to the interference substances and extremely small quantity of $N\textsuperscript{-}$-MH. Thus, we tried to make our pervious method (Hayashi et al., 1987) more simple and reliable.

**Materials and Methods**

Chemicals. The chemicals used were of analytical grade and commercially available unless specifically stated otherwise. $N\textsuperscript{-}$-MH was purchased from Sigma (St. Louis, MO., USA). The water used was distilled and ion exchanged.

Mobile phase. KH$_2$PO$_4$ (20 mM), sodium 1-octane sulfonate (2.7 mM) for HPLC. The solution was filtered through a 0.45-µm aqueous filter (Millipore, Bedford, MA, USA).

Fluorescent reagent. Mercaptoethanol (26 mM), 12 mM orthophthalaldehyde (OPA), and 0.12 M methanol in borate buffer (0.4 M, pH 10.6). Orthophthalaldehyde was first solubilized with methanol and mixed with the buffer.

Formaldehyde. Formaldehyde (2.5 M) in borate buffer (0.4 M, pH 10.6).

Equipment. A Shimadzu LC-6A liquid chromatograph equipped with Inertsil ODS-2 column (4.6×250 mm), with a guard column (Shimadzu guard column ODS, 1 cm long, 1 ml volume capacity) was used. The fluorescent product was monitored in a Shimadzu Fluromonitor using an excitation wavelength of 348 nm and an emission wavelength of 460 nm, and peak height was quantified by the method of external standardization using $N\textsuperscript{-}$-MH. The fluorescent intensity was recorded on a linear recorder. The column, the mixing coil and the reaction coil were attached to an oven (45°C). The column eluate was first mixed with the formaldehyde reagent in the mixing coil (20 cm), and it was then mixed with OPA reagent in the reaction coil (200 cm). A schematic diagram of post column fluorescence derivatization is shown in Fig. 1.

![Fig.1. Schematic diagram of post-column fluorescence derivatization system.](image-url)
The mobile phase was pumped at the rate of 1.2 ml/min. Formaldehyde reagent and OPA reagent were pumped at the rate of 0.6 ml/min.

Sample preparation. Commercial chicken food (about 0.5 g) and chicken excreta (about 2 g wet) were hydrolyzed in 8 ml of 6 N HCl at 110°C for 20 hours in autoclave. The hydrolysates were cooled and filtered through filter paper and made up to 50 ml with water, and 2 ml of the hydrolysate was applied to the cation-exchange resin column. The resin (Dowex 50 × 8, 200–400 mesh, pyridine form) was packed by gravity to give a bed height of 6 cm and a column volume of 2 ml. The column had previously been equilibrated with 0.2 M pyridine. After eluting most of the acidic and neutral amino acids with 30 ml 0.2 M pyridine, Nansible-MH was eluted with 20 ml 1 M pyridine. This step was necessary to remove the amino acids, which may interfere with the Nansible-MH analysis. The eluent was then dried using a rotary evaporator and the residue was dissolved in 1 ml of the HPLC mobile phase, filtered through a 0.45-µm aqueous filter, and 50 µl of this solution was injected in HPLC in order to measure the Nansible-MH fraction.

Results and Discussion

The major modification in the present method was as follows: Hydrolysate was directly applied to a resin column without evaporation of HCl, which was previously a time-consuming step. It took about 30 min to evaporate the HCl for every sample. The HPLC column and the concentrations of mobile phase and formaldehyde were also changed as described in the materials and methods. The volume of 0.2 M pyridine was increased up to 30 ml to ensure eluting of acid and neutral amino acid from the resin column.

A chromatogram of standard Nansible-MH (1.04 nmol/10 µl) is shown in Fig. 2 (A), the

![Fig. 2](image-url)

Fig. 2. (A)-Chromatogram for 50 µl standard Nansible-MH (5.2 nmol), (B)-Chromatogram for 50 µl of the mixed sample of Nansible-MH standard (0.26 nmol) solution and chicken excreta sample. 1, histidine peak; 2, Nansible-MH peak and (C)-Chromatogram for 50 µl chicken excreta sample. 1, histidine peak; 2, Nansible-MH peak.
retention time was about 18 min and the recovery (peak height of the present method/peak height when N$^\text{\textdagger}$-MH standard was directly injected in HPLC) was 99±0.6%. Fig. 2 (B) shows a clear separation of N$^\text{\textdagger}$-MH in the mixed sample of standard and hydrolyzed chicken excreta. The recovery of N$^\text{\textdagger}$-MH added to the excreta was 98±2.0%. These results show that HCl-containing samples can be directly applied to the ion-exchange resin column. In the previous method (Hayashi et al., 1987), we had to remove HCl before applying the sample into the cation-exchange resin column.

We measured N$^\text{\textdagger}$-MH content 3 times using an excreta sample by the present method and the value of 20.6±0.15 μmol/day/bird was given as shown in Fig. 2 (C). Similarly, the value of 129±7 nmol/g was given for a feed formulated as our basal diet in which animal source feed was not contained.

The present modification provides a faster and reliable method to measure N$^\text{\textdagger}$-MH in chicken feed and excreta.

References


