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Measurement of 1- and 3-methylhistidine in human urine by ultra performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

Background: Determination of 1-methylhistidine (1-MH) and 3-methylhistidine (3-MH) is important to monitor muscle protein catabolism. Here, an ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method for quantification of 1-MH and 3-MH in human urine is described.
Methods: Urine samples were prepared by dilution with water after addition of isotopic internal standard. The samples were chromatographed on a SB-aq (2.1 × 50 mm, 1.8 μm) column with acetonitrile — 0.1% formic acid in water (2:98, v/v) as mobile phase. Mass spectrometric detection was performed on a triple quadrupole mass spectrometer using positive electrospray ionization (ESI). 1-MH and 3-MH were monitored by the following transitions: 1-MH, *m/z* 170.1→ *m/z* 126.1; 3-MH, *m/z* 170.1→ *m/z* 124.1.
Results: For 1-MH and 3-MH, calibration curves were linear over the concentration range of 5–500 nmol/ml. The lower limit of quantification was 5 nmol/ml. The accuracy was within 85%–115% and precision was <15%. 1-MH and 3-MH were proved to be stable under different storage and processing conditions. In addition, the detection was independent of matrix effect.
Conclusion: This rapid and specific UPLC/MS-MS method is suitable for the determination of urinary 1-MH and 3-MH.

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1. Introduction

Nutritional support to the patients is of critical importance in clinical treatment. It has been recognized that regulation of protein turnover is the central aspect of overall metabolic and physiological homeostases. A full understanding of protein turnover requires knowledge of both synthesis and breakdown. The excretion of urinary 3-methylhistidine (3-MH) has been used to estimate the rate of muscle protein breakdown for >30 years [1–3].

3-Methylhistidine is formed by post-translational methylation of histidine residues of the muscle actin and myosin [4,5]. On degradation of these proteins, the released 3-MH is neither reutilized for protein synthesis [6] nor metabolized, but is excreted in the urine [7]. It has been demonstrated that urinary 3-methylhistidine originates mainly from skeletal muscle, with minor contributions from the skin and gastrointestinal tract [8,9]. Therefore, measurement of 3-MH in urine is considered a useful non-invasive technique to assess turnover of muscle protein and its degradation during several clinical conditions, e.g. malnutrition, sepsis and trauma [1–3,10–12]. However, in clinical studies some factors may compromise the use of urinary 3-MH because proteolysis is not the only source of 3-MH. Dietary intake of meat, an exogenous source of 3MH, may result in increased urinary excretion of 3-MH [13]. Endogenic 3-MH can be distinguished from the exogenic one by detecting another methylated derivative of histidine, 1-methylhistidine (1-MH), which is not formed in humans, but it is common in other animals. The 1-MH content in human urine correlates well with dietary 3-MH and may be used as an objective marker of exogenous 3-MH intake [14,15]. Therefore, the level of 1-MH should be determined together with that of 3-MH when monitoring muscle protein catabolism.

Traditional techniques that have been used for the determination of urinary 1-MH and 3-MH include high-performance liquid chromatography (HPLC) [16–18], gas chromatography–mass spectrometry (GC–MS) [19,20] and capillary electrophoresis (CE) [21,22]. However, these methods often require laborious derivatization and time-consuming analytical procedure. Recently, another powerful technique, HPLC–MS/MS, has been applied to amino acid analysis [23,24], but an examination of the literature reveals that few references are available for the quantification of 1-MH and 3-MH.

2. Materials and methods

2.1. Chemicals and reagents

1-MH, 3-MH and 3-d_3-MH (internal standard) were purchased from Sigma-Aldrich (St.Louis, MO). Chemical structures of the compounds were shown in Fig. 1. HPLC grade acetonitrile, formic acid (98%) and hydrochloric acid (37%) were from Fisher-Scientific

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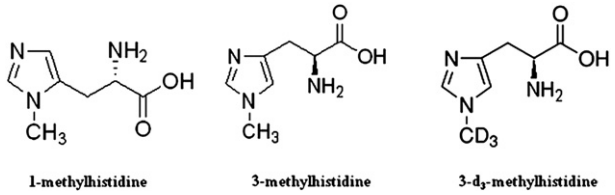

Fig. 1. Chemical structures of 1-MH, 3-MH and 3-d3-MH (IS).

(Rutherford, NJ). Purified water was produced in our laboratory by Thornton 200CR pure UV system (Mettler Toledo, OH). Other chemicals were of analytical grade or better.

2.2. Chromatography

The UPLC unit consisted of an Acquity UPLC® separation module (Waters, Milford, MA) controlled by the Masslynx® software. Separations were performed on a SB-Aq analytical column (2.1×50 mm, 1.8 μm) (Agilent, Santa Clara CA) at room temperature. The mobile phase was composed of acetonitrile — water with 0.1% formic acid (2:98, v/v). The flow rate was 0.4 ml/min and the injection volume was 5 μl. The analytical run time for each sample was 1.0 min.

2.3. Mass spectrometry

A Xevo TQ triple quadrupole mass spectrometer (Waters) equipped with electrospray ionization source (ESI) was used for detection. The mass spectrometer was performed in positive ionization mode. The capillary voltage and cone voltage were set at 3000 and 20 V, respectively. The ESI source was operated at a temperature of 150 °C with ultrahigh-purity nitrogen as cone gas (50 l/h). The desolvation temperature was 350 °C and the gas flow rate was set at 900 L/h. With argon as collision gas (3.6^{-3} mBar), the collision energy was set at 20 eV for 1-MH, 3-MH and 3-d_3-MH. Multiple reactions monitoring (MRM) was applied to detect the analytes by the following ion transitions: 1-MH, *m/z* 170.1→126.1; 3-MH, *m/z* 170.1→124.1; and 3-d_3-MH (IS), 173.1→127.1. Product ion spectra of the analytes were shown in Fig. 2.

2.4. Stock and working solutions, calibration curve and quality control (QC) samples

The stock solutions of 1-MH and 3-MH (10 μmol/ml) were prepared in duplicate, one for calibration curve and the other for quality control (QC) samples, by dissolving the accurately weighed standards with water. The stock solution of IS (5 μmol/ml) was prepared and then diluted with water to achieve a working solution at the concentration of 200 nmol/ml, which was used in sample preparation. The calibration curve and QC samples in urine were prepared by diluting the corresponding stock solutions of 1-MH and 3-MH with freshly pooled human urine from 6 individuals. The added concentrations of 1-MH and 3-MH in the calibration samples were 5, 10, 25, 50, 100, 250, 500 nmol/ml. For QC samples, the added concentrations were 15, 200, 400 nmol/ml. Note: all the solutions and the spiked urine samples were stored at −30 °C.

2.5. Sample preparation

To each 25-μl volume of urine sample, 25 μl of IS working solution (3-d_3-MH, 200 nmol/ml) and 2.0 ml of water were added. After being vortex-mixed for 30 s, 100 μL of the diluted sample was transferred to a micro-spin filter tube (0.2 μm nylon, Alltech) and centrifuged at 13,000 rpm for 1 min. Finally, 5 μl of the filtered sample was injected into UPLC–MS/MS system.

2.6. Method validation

The method was validated for specificity, linearity, lower limit of quantification (LLOQ), accuracy, precision, extraction recovery, stability and matrix effect according to the guidelines issued by Food and Drug Administration (FDA) and European Medicines Agency (EMA) [25,26].

For specificity, blank urine samples from six individuals were tested to verify that interfering substance was not observed at the retention time of the analytes. For linearity, 7-point calibration curves including blank sample were analyzed on three independent batches. LLOQ was defined as the lowest non-zero concentration on the calibration curve and five aliquots of LLOQ samples were analyzed on 3 independent batches. Inter- and intra-batch CVs and accuracy were determined by assessing QC samples at low, medium and high concentration levels (i.e., 15, 200, and 400 nmol/ml) on three independent batches. Extraction recovery was evaluated by comparing the peak area of extracted low, medium and high QC samples with the peak area of pure solutions at the same concentration levels. Stability of 1-MH and 3-MH in human urine was investigated by comparing the low, medium and high QC samples (n=5) undergoing a variety of storage and processing conditions, with those freshly prepared QC samples at the same concentration levels. For the freeze–thaw stability, the QC samples were analyzed after three freeze–thaw cycles (frozen at −30 °C and thawed at room temperature, each cycle at an interval of 12 h). The short-term stability (bench-top stability) was investigated by assessing the QC samples after they were kept at room temperature (25 °C) for 24 h. The stability of processed samples was investigated after the processed QC samples were kept at autosampler (10 °C) for 12 h. The stock solution stability of 1-MH, 3-MH and IS was tested by comparing fresh solutions with the solutions stored at −30 °C for 1 months.

Matrix effect was evaluated using blank urine samples from 6 individuals. For each analyte and IS, the matrix factor (MF) was calculated in each lot of urine. This test included 3 groups of samples: (A) blank urine samples (n=5); (B) blank urine spiked with analytes at the concentration of 3 times the LLOQ (i.e., 15 nmol/ml for 1-MH and 3-MH after extraction (n=5); (C) Pure solutions of the analytes (n=5) at the equivalent concentration levels. MF was calculated based on the following equation: MF (%)=(B−A)/C×100. (Note: Baseline of blank urine should be subtracted because 1-MH and 3-MH are endogenous substances). The MF of IS was evaluated at the concentration of 200 nmol/ml. A MF value of 100% indicated that the detection was not affected by matrix. Accordingly there was signal enhancement if the value was >100% and signal suppression if the value was <100%. The IS normalized MF was calculated by dividing the MF of the analyte by the MF of the IS. The CV of the IS-normalized MF calculated from the 6 lots of urine should not be >15% [26].

2.7. Investigation of hydrolysis of N-acetyl-3-methylhistidine in human urine

It is reported that 3-MH has 2 forms: free and N-acetyl-3-MH [8]. The N- acetylated 1 accounts for 80%–90% of total urinary excretion in animals, particularly in rats [27,28]. Therefore, the N-acetyl derivative must be converted to the free one by hydrolysis when analyzing the rat urine samples. However, the hydrolysis step may be not required for human samples because less than 5% of 3-MH excreted as N-acetyl form [7]. Unfortunately, the literature was not consistent about the hydrolysis step because some publications did [20] but most not [16–19,29].

In this paper the necessity of hydrolysis step was evaluated by testing the urine samples from 6 individuals. In the hydrolysis group, after addition of 25 μl of IS buffer, 25 μl of each urine sample (n=6) was hydrolyzed by adding an equal volume of 10 mol/l HCl and incubating at 110 °C for 30 min. The urine samples were then

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