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Short communication

Standardization of acid hydrolysis procedure for urinary 3-methylhistidine determination by high-performance liquid chromatography

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Abstract

The N-acetylated form of N-methylhistidine (3-methylhistidine, 3-meH), a non-invasive marker of proteolysis, accounts for 80–90% of total 3-meH excretion (acetylated+non-acetylated 3-meH) in the rat. To determine total 3-meH excretion, samples require acid hydrolysis prior to determination by high-performance liquid chromatography. This study evaluated the stability of 3-meH at various times and temperatures of hydrolysis and determined the optimal conditions for hydrolysis of samples. Increasing temperature (120°C) results in significant degradation of 3-meH with no appreciable change in concentration being noted at 80°C. Hydrolysis at 100°C for 1.5 to 4 h or 80°C for 8 to 12 h is recommended for determining total 3-meH concentrations in rat urine.

Keywords: 3-Methylhistidine; Actin; Myosin

1. Introduction

N-Methylhistidine (3-methylhistidine, 3-meH) is an integral component of actin and myosin that is of considerable interest to scientists studying protein metabolism. It is formed by post-translational modifications of certain histidine residues of the polypeptide chain. During protein degradation, it is released and cannot be reutilized for protein synthesis because specific tRNA for this amino acid is lacking [1]. Studies in both animals and humans have demonstrated that 3-meH is not significantly metabolized and is excreted unchanged in the urine [1,2]. As a result, the urinary excretion of 3-meH has been used as a noninvasive marker of proteolysis [3–6].

A rapid and sensitive high-performance liquid chromatographic method (HPLC) has been developed for the determination of human plasma and urinary 3-meH [7]. However, 3-meH exists in two forms: N-acetyl-3-meH and the parent compound. The site of acetylation is not known, but presumably is the liver [8]. In the human adult, less than 5% of urinary 3-meH exists in the acetylated form [2]. However, in the rat, the N-acetylated form may account for the majority of total urinary 3-meH excretion, varying from 80% to 90% of total 3-meH [6,8]. Therefore, a hydrolysis step in sample prepara-

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tion is necessary prior to determination of 3-meH. Unfortunately, the literature is not consistent and is confusing as to the most appropriate temperature and duration for this acid hydrolysis step [3–6]. This may be particularly pertinent in the event 3-meH is modestly heat labile. This would result in two divergent processes influencing total (deacetylated) 3-meH concentrations. Increasing measured total 3-meH will occur due to hydrolysis of N-acetyl-3-meH with subsequent degradation of free compound possibly occurring when higher temperatures or longer periods of hydrolysis are utilized.

The purpose of this study was to document the stability of 3-meH during acid hydrolysis and to determine the optimal temperature and duration of acid hydrolysis for measurement of total 3-meH in rat urine. Our intent was to standardize acid hydrolysis procedures for future studies examining urinary 3-meH in the rat.

2. Experimental

2.1. Materials

Fluorescamine, 3-methylhistidine, L-histidinol, 3-(N-morpholino)propanesulfonic acid (MOPS), sodium hydroxide and boric oxide were obtained from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade) and perchloric acid (PCA) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Monobasic potassium phosphate (KH_2PO_4) and dibasic sodium phosphate (Na_2HPO_4) were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. HPLC instrumentation

The HPLC system consisted of two LC10AD solvent delivery pumps, SCL-10A system controller, CR501 integrator, RF535 fluorescence monitor and SIL-10A automatic sample injector (Shimadzu Corporation, Kyoto, Japan). Separation was obtained using a Dynamax C_{18} 8-μm (250×4.6 mm I.D., 80 Å pore size) column (Rainin, Woburn, MA, USA) with an Adsorbosphere C_{18} 10-μm universal guard column (Alltec Associates, Deerfield, IL, USA).

2.3. Chromatographic conditions

Resolution was obtained using an isocratic system of acetonitrile and water (20.5:79.5, v/v) containing a 0.01 *M* phosphate buffer (272 mg of KH_2PO_4 and 1136 mg of Na_2HPO_4 per liter of water) and 250 μl diethylamine per liter. The mobile phase was recirculated at a flow-rate of 1.6 ml/min. The fluorescence detector was set at an excitation wavelength of 365 nm and emission wavelength of 460 nm.

2.4. Standard and reagent preparations

Standard stock solutions of 3-meH, histidine and L-histidinol were prepared by adding 10 mg of each compound to 100 ml of deionized water (100 mg/ml). Eight serial dilutions of 3-meH to achieve concentrations ranging from 0.39 mg/ml to 100 mg/ml were used for the standard curve. MOPS buffer (0.4 *M*) was prepared by adding 20.93 g MOPS to 250 ml of 2.4 *M* sodium hydroxide solution. Boric oxide buffer was made by mixing 5.6 g boric oxide in 250 ml of deionized water with adjustment to pH 12.2 with 12 *M* sodium hydroxide. A 100-mg quantity of fluorescamine was added to 250 ml of acetonitrile for preparation of fluorescamine solution.

2.5. Sample preparation

To determine the effect of temperature and duration of hydrolysis on deacetylated (total) 3-meH concentration, pooled rat urine samples (n=4–7) were heated at 80, 100, or 120°C for 0, 30, 60, 90 min, 2, 3, 4, 6, 8 and 12 h. A 12.5 μg/ml 3-meH control solution (n=4) was heated at 80, 100, or 120°C for 0, 2, 4, 8, 12 and 20 h to ascertain heat stability of 3-meH. A 50-μl volume of PCA was added to 200 μl of sample or control in 100×13 mm screw top glass culture tubes with Teflon lined caps and vortex-mixed for 5 s and heated as described.

Derivatization of 3-meH was performed according to a modification of the methods of Wassner et al. [7]. Following acid hydrolysis, 750 ml of deionized water were added, and tubes were inverted and vortex-mixed for 5 s. The sample was transferred to a 1.5-ml microcentrifuge tube and centrifuged at 3500 *g* for 5 min. A 75-μl aliquot of diluted sample

was placed in a 100×13 mm screw-top glass culture tube with 250 μl boric oxide buffer and vortex-mixed. Fluorescamine (250 μl) was added, vortex-mixed for 5 s, and allowed to stand for 5 min. PCA (35 μl) was then added, and samples were vortex-mixed and heated at 80°C for 1 h. Upon cooling to room temperature, 100 μl of MOPS buffer was added and the samples were inverted, vortex-mixed, and transferred to autosampler vials. A 100-μl aliquot of each sample was injected onto a 500-μl sample loop for analysis.

2.6. Statistics

Continuous data were analyzed by one-way analysis of variance (ANOVA) with post-hoc pairwise analysis using Duncan's multiple range test. A probability value of 0.05 or less was defined as statistically significant. Continuous data were given as mean ±S.D.

3. Results

The acetonitrile–water mobile phase provided for excellent resolution of 3-meH. Possible interfering substances (L-histidinol and histidine) were evaluated and were resolved from 3-meH. A representative chromatogram from a urine sample is given in Fig. 1. The peak of interest resolved at approximately 15 min with a run time of about 22 min.

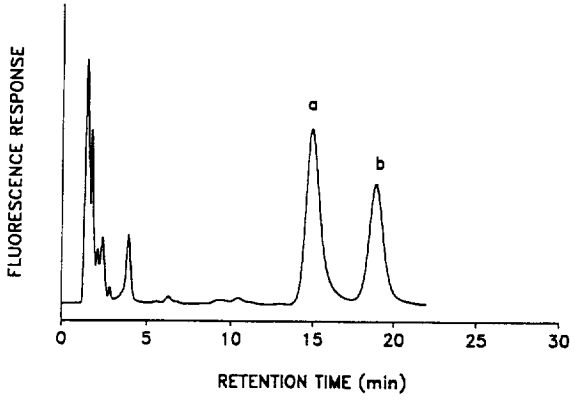

Fig. 1. Representative reversed-phase chromatogram of urine sample. Peaks: a=3-methylhistidine; b=endogenous unknown.

Table 1
Accuracy and precision of 3-methylhistidine determinations in standard solutions

Actual concentration[a] (μg/ml)	Within-day		Between-day	
	Observed concentration (μg/ml)	C.V. (%)	Observed concentration (μg/ml)	C.V. (%).
80	78.46	1.49	79.08	1.79
20	19.19	4.16	19.29	1.59
5	5.06	3.81	4.92	4.70
0.625	0.676	2.56	0.653	4.41

[a] $n=5$ for both with-in day and between-day determinations.

Standard curves were linear over the concentration range used (0.39 to 100 mg/ml) with coefficients of determination greater than 0.999. Table 1 describes the precision and accuracy of the assay. Between-day coefficients of variation and percent error were less than 5% for all concentrations. Within-day coefficients of variation and percent error ranged from 1.5 to 4.2% and 1.1 to 8.2%, respectively.

The stability of a standard concentration of 3-meH (12.5 mg/ml) during acid hydrolysis over time at three different temperatures is depicted in Fig. 2. There was no statistically significant change in concentration during acid hydrolysis at 80°C and

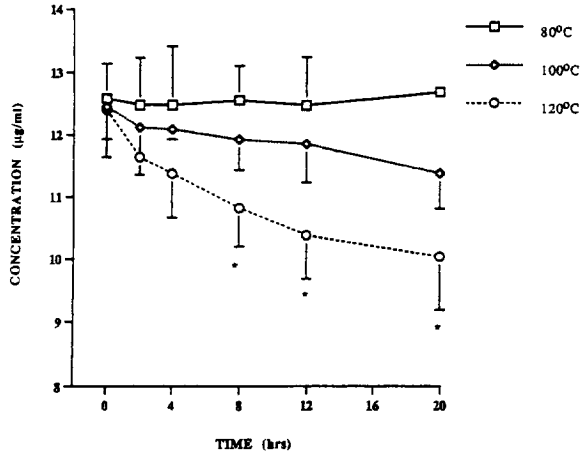

Fig. 2. Change in concentration of control 3-meH sample at 12.5 μg/ml over time at three different temperatures. Statistically significant decreases in 3-meH concentration from baseline were found at 8, 12 and 20 h ($P \leq 0.05$) at 120°C.

100°C for the 20-h study period; however, a significant reduction in concentration from baseline was found at 8, 12 and 20 h at 120°C ($P \leq 0.01$; Fig. 2). Concentrations were significantly lower using the 120°C temperature compared to the 80°C and 100°C temperatures by 4 h and 8 h, respectively ($P \leq 0.05$). Hydrolysis at 100°C resulted in concentrations that were not statistically different from the 80°C at any time point.

Fig. 3 illustrates the changes in total 3-meH concentration in pooled rat urine over 12 h of acid hydrolysis at the different temperatures. Peak concentrations were achieved at 1, 1.5 and 8 h for 120, 100 and 80°C, respectively. The 120°C temperature showed a significantly lower peak 3-meH concentration ($P \leq 0.05$) than the other temperatures (10.56 ± 0.51, 11.67 ± 0.71, and 12.16 ± 0.60 μg/ml for 120, 100 and 80°C, respectively). No statistically significant difference in peak 3-meH concentrations was found between the 100 and 80°C groups. Mean 3-meH concentration was significantly decreased from peak 3-meH by 2 h (9.73 ± 0.15 mg/ml) during hydrolysis at 120°C ($P \leq 0.05$). Hydrolysis at 100°C resulted in mean concentrations which were not significantly different at 1.5, 2, 3 and 4 h. Mean concentrations were significantly decreased by 6 h at this temperature ($P \leq 0.05$). Hydrolysis at 80°C resulted in mean concentrations which were not significantly different at 6, 8 and 12 h.

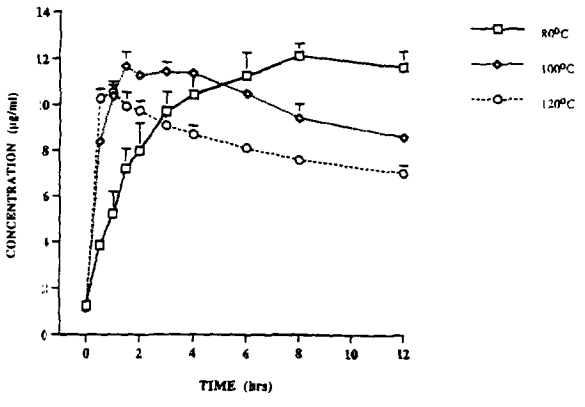

Fig. 3. Rate of deacetylation of free 3-meH from total urinary 3-meH during acid hydrolysis at three different temperatures over time. The highest temperature (120°C) had a significantly lower peak ($P \leq 0.01$) than the other two temperatures which were not different from each other. The peak was achieved by 1.5 h of acid hydrolysis at 100°C and by 8 h at 80°C.

4. Discussion

The present study provides evidence for the need to standardize the acid hydrolysis procedure for determination of total (acetylated and nonacetylated) urinary 3-methylhistidine excretion in rats. A significant, but variable, portion of urinary 3-meH is acetylated in rats [6]. Therefore, measurement of total 3-meH following acid hydrolysis is essential when using 3-meH as a marker for assessing protein catabolism in rats. Various times and temperatures for acid hydrolysis of rat urine samples have been reported in the literature [3–6]; however, it is known that acid hydrolysis may degrade amino acids [9]. Therefore, it is important to characterize the changes which occur in 3-meH concentrations while varying the time and temperature of acid hydrolysis. Equally important is to simultaneously optimize deacetylation and minimize degradation of 3-meH during the acid hydrolysis procedure.

Our results reveal that acid hydrolysis degrades 3-meH with higher temperatures resulting in greater degradation. Incubation of urine samples at 120°C resulted in significantly lower total 3-meH concentrations and caused significant degradation of 3-meH controls over time. No appreciable degradation in 3-meH controls was noted during the 20-h cook time at temperatures of 80°C. Although there was no statistically significant decline in 3-meH control at 100°C, concentrations decreased from 12.45 ± 0.45 μg/ml to 11.39 ± 0.50 μg/ml ($n=4$) over the 20-h cook time.

Determining the optimal time and temperature for deacetylation of rat urine samples revealed that temperatures equal to or above 120°C should be avoided even during short periods of incubation. Hydrolysis of rat urine samples at either 100°C for 1.5 to 4 h or 80°C for 8 to 12 h results in optimal total 3-meH concentrations. Use of longer cook times at 100°C is not recommended since some degradation of 3-meH does occur at this temperature. Longer hydrolysis periods (up to 20 h) at 80°C can be utilized based upon the stability of 3-meH controls over this time. These standardized procedures

should be utilized in all future studies that require determination of total 3-meH concentration.

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