ORIGINAL ARTICLE

Isotopic decay of urinary or plasma 3-methylhistidine as a potential biomarker of pathologic skeletal muscle loss

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Abstract

Background Skeletal muscle loss accompanying aging or cancer is associated with reduced physical function and predicts morbidity and mortality. 3-Methylhistidine (3MH) has been proposed as a biomarker of myofibrillar proteolysis, which may contribute to skeletal muscle loss.

Methods We hypothesized that the terminal portion of the isotope decay curve following an oral dose of isotopically labeled 3MH can be measured non-invasively from timed spot urine samples. We investigated the feasibility of this approach by determining isotope enrichment in spot urine samples and corresponding plasma samples and whether meat intake up to the time of dosing influences the isotope decay.

Results Isotope decay constants (k) were similar in plasma and urine, regardless of diet. Post hoc comparison of hourly sampling over 10 h with three samples distributed over 10 or fewer hours suggests that three distributed samples over 5–6 h

of plasma or urine sampling yield decay constants similar to those obtained over 10 h of hourly sampling.

Conclusion The findings from this study suggest that an index of 3MH production can be obtained from an easily administered test involving oral administration of a stable isotope tracer of 3MH followed by three plasma or urine samples collected over 5–6 h the next day.

Keywords Cachexia · Sarcopenia · Human · Tracer

1 Introduction

Unintentional loss of skeletal muscle occurs in most individuals as they age [1–5] and at accelerated rates during periods of inactivity [6–13] or affliction with certain diseases, such as cancer [14–21]. This loss of muscle mass is associated with reduced functional capacity [4, 22, 23], metabolic deterioration [24, 25], and poor prognosis [19, 26, 27]. Cancer-associated skeletal muscle loss (cancer cachexia) is common and inauspicious, with approximately 20 % of cancer deaths attributed to cachexia [28]. Likewise, loss of skeletal muscle mass has been associated with mortality in healthy, community-dwelling older adults as well as those living in nursing homes [27].

These negative associations suggest that those at risk for, or already experiencing, muscle loss may benefit from preventive, or ameliorative, measures [29, 30]. This notion is supported by recent studies showing that prevention of cancer cachexia in tumor-bearing mice prolongs survival [31, 32]. However, a significant obstacle to the translation of such a strategy in humans is the retrospective nature of the diagnosis of age- and disease-associated muscle atrophy. That is, sarcopenia and cachexia describe the *existence* of muscle mass loss, which is a manifestation of a process initiated at some prior point in time. This is an important practical point, as sarcopenic or cachectic individuals may be less sensitive to

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the anabolic effects of interventions than healthier individuals [33–38], making it difficult to reverse the muscle loss. It seems reasonable that those susceptible to harmful muscle loss during aging, inactivity, or disease might respond more favorably to interventions if they could be initiated before significant loss occurs. However, there are currently no means of determining which individuals will experience or are in the early stages of harmful muscle loss during aging, inactivity, or disease, hindering earlier intervention with anabolic therapies.

3-Methylhistidine (3MH) is one potential biomarker for susceptibility to muscle mass loss. Formed by the post-translational methylation of specific histidine residues in the myofibrillar proteins actin and myosin [39, 40], it is released when these proteins undergo proteolysis [41, 42]. As it is not capable of charging tRNA [42, 43], it is not reutilized for protein synthesis and is quantitatively excreted in the urine [44]. Because the concentration of 3MH in skeletal muscle proteins is constant throughout life [41], it is well-suited for the study of age-, illness-, disease-, or inactivity-induced changes in muscle protein turnover [41, 42, 44–48]. The main hindrances to the widespread use of 3MH as a biomarker are the need to abstain from meat for 3 days prior to sample collection (as 3MH is present in meat), and, when urine is sampled, the need for 24-h urine collection. In this study, we sought to avoid these limitations by using a stable (i.e., nonradioactive) isotope-based strategy. We hypothesized that the terminal portion of the isotope decay curve in plasma and urine following ingestion of a single oral dose of methyl-d_3-3MH (D-3MH) should reflect skeletal muscle protein turnover rather than absorption of 3MH from the diet and that this decay can be measured noninvasively from timed spot urine samples obtained during this period. Here, we investigated the feasibility of this approach by determining if isotope enrichment in spot urine samples is similar to that in corresponding plasma samples and whether meat intake up to and including the time of dosing influences the slope of the terminal portion of the isotope decay curve. We report that meat ingestion at the time of tracer dosing did not affect overall D-3MH isotope enrichment decay 13–22 h afterward in plasma or urine, although it did slightly increase variability in estimated decay constants. Further, isotopic decay was similar in plasma and urine. These results suggest that isotopic decay of urinary or plasma 3MH can be used as a biomarker to assess muscle protein breakdown without the need for quantitative urine collection or several days of abstinence from meat.

2 Materials and methods

Clinical protocol Informed written consent, which was approved by the Institutional Review Board of the University of Texas Medical Branch, was obtained from all volunteers prior to any study-related procedures. Thirteen apparently healthy male subjects were screened at the Institute for Translational Sciences Clinical Research Center (CRC) at the University of Texas Medical Branch in Galveston, TX, USA. Exclusion criteria included presence of any known serious illness (including type II diabetes controlled by medication), uncontrolled hypertension, glomerular filtration rate of less than 60 mL/min/1.73 m^2, history of recurrent gastrointestinal bleeding, and inability or unwillingness to provide informed consent. Of the 13 healthy subjects screened, 9 successfully completed the clinical protocol ($N=9$, 52±18 years (mean±SD), 175±11 cm, 82±13 kg; body mass index (BMI), 26±2 kg/m^2; glomerular filtration rate, 72±12 mL/min/1.73 m^2; 100 % Caucasian). Lean body mass (LBM) of each subject was determined using dual energy X-ray absorptiometry. Each subject was studied on two occasions: once following consumption of a meat-containing evening meal and once following consumption of a meatless evening meal. Subjects were asked to refrain from strenuous activity the day prior to each study. Meal order was randomly determined and macronutrient proportions of the meat and meatless meals consumed at the time of tracer dosing were matched for macronutrient composition, as were the meatless meals consumed for breakfast and lunch on the day of dosing as well as on the following day, when blood and urine sampling occurred (Table 1). With each evening meal, the subject ingested a dose (8.54±0.11 mg, 49.6 μmol) of D-3MH mixed into a small volume (~100 mL) of diet soda. Following ingestion of the D-3MH-containing soda, the cup was rinsed with additional soda and the rinse consumed in order to ensure complete ingestion of the tracer dose.

The D-3MH used in this investigation was purchased from Cambridge Isotope Laboratories, Andover, MA, USA (Cat# DLM-2949-SP, Lot# PR-19852) and was 99.1 % isotopically pure. The D-3MH doses were prepared ahead of time by dissolving the total amount in an appropriate volume of distilled water and then aliquoting this into individual doses. Doses were stored at −80 °C until shortly before use. In this way, each subject received the same amount of tracer for each trial.

The morning after each test meal (12 h post-dosing), the subject voided, discarding any urine produced overnight. After an indwelling catheter was placed in the antecubital vein of each subject's nondominant arm to facilitate blood sampling, hourly blood and urine sampling commenced, continuing for the next 10 h. During the 10-h sampling period, all subjects were encouraged to drink liberally in order to facilitate spot urine collections. The volunteers were served a meat-free breakfast, lunch, and dinner. At the end of the sampling period, the subjects were discharged from the CRC. For all subjects, the test procedure was repeated with the other test meal 10–14 days later. Blood was collected into ethylenediaminetetraacetic acid-containing collection tubes. Blood collection tubes and urine samples were immediately placed on ice until plasma could be separated and urine aliquoted. Plasma and urine samples were stored at −80 °C until both studies were completed in all subjects. After the final subject had been studied, plasma and urine

Table 1 Energy and macronutrient composition of the study diet. Values are mean±SD

	Day 1 (breakfast, lunch, dinner)		Day 2 (breakfast, lunch)	
	Meat	Meatless	Meat	Meatless
Energy (kcal/kg LBM)	44.3±2.1	43.7±3.6	26.2±3.3	23.9±2.3
Fat (g/kg LBM)	1.54±0.08	1.51±0.14	0.76±0.18	0.69±0.05
Carbohydrate (g/kg LBM)	6.17±0.39	6.09±0.47	3.84±0.45	3.63±0.43
Protein (g/kg LBM)	1.74±0.07	1.68±0.18	1.13±0.07	1.04±0.11

samples were shipped on dry ice for isotopic analysis by gas chromatography–mass spectrometry (GC-MS) by Metabolic Technologies, Inc. (2625 North Loop Drive, Suite 2150, Ames, IA, USA).

D-3MH decay Isotopic enrichment of D-3MH in plasma and urine was determined using GC-MS as described previously [49, 50]. Briefly, samples (plasma or urine) were acidified, absorbed onto a cation-exchange column, and eluted using ammonium hydroxide. Eluates were then dried and derivatized for GC-MS. The D-3MH enrichment was measured as the ratio of peak areas at 241 and 238 m/z using an Agilent GC/MS Model 6890/5973. GraphPad Prism (version 5.02 for Windows, GraphPad Software, San Diego, CA, USA; www.graphpad.com) was used to generate exponential decay curves ($E_t = E_0 e^{-kt}$) from plasma and urine D-3MH enrichment versus time data, from which the decay rate (k) was used as an indicator of endogenous 3MH appearance where E_t is the enrichment of D3MH at time t following bolus tracer ingestion, and where E_0 is the theoretical D3MH enrichment at the beginning of decay. As a practical consideration, goodness of fit (R^2) and k for different durations of sampling over 5–10 h were also examined using the data from the meatless diet study.

Statistical analysis Isotopic decay constants from the single-exponential fit of the isotopic decay data in plasma and urine for each diet were compared using an analysis of variance approach including effects of sample type (urine vs. plasma) and dosing meal (meat-containing, meatless), as well as their potential interaction, using Prism. R^2 and k for different durations of sampling (5–10 h) and sampling frequency (hourly vs. three approximately equally distributed samples) were compared using a two-way repeated measures analysis of variance in Prism. Decay constants from young and old subjects were compared using two-tailed t tests in Prism. A P value of ≤0.05 was considered statistically significant.

3 Results

Urine and plasma D-3MH enrichment decay Enrichments of D-3MH in urine and plasma were similar following ingestion of a meat-containing (Fig. 1a) or a meatless (Fig. 1b) meal at the time of tracer dosing. Enrichment decays (k) over the 10 h of sampling were similar in urine and plasma and not affected by diet, whether expressed in units of per hour (Fig. 1c) or per hour per kilogram per LBM (not shown).

Effect of sampling duration and frequency on D-3MH enrichment decay Urinary k values varied significantly over 5–10 h of sampling (Fig. 2a, $P < 0.0001$) but were not significantly affected by sampling method (hourly vs. distributed, Fig. 2a; $P = 0.52$). In plasma, k values were not significantly different with sampling durations ranging from 5 to 10 h (Fig. 2b, $P = 0.45$) or with hourly vs. distributed sampling (Fig. 2b, $P = 0.95$).
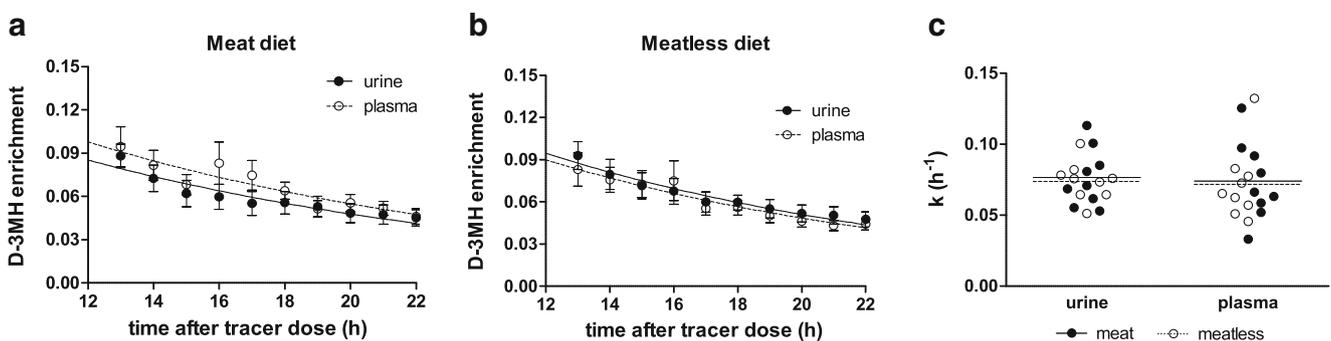

Fig. 1 Isotopic enrichment of D-3MH in urine and plasma the day following oral stable isotope tracer administration when meat was (**a**) or was not (**b**) consumed along with the oral tracer. Corresponding enrichment decay constants (k) are presented in **c**. In **a** and **b**, symbols and *error bars* represent mean and standard error, respectively. In **c**, *lines* indicate group (i.e., meat, meatless) mean values

Fig. 2 Decay (k; **a**, **b**) and goodness-of-fit (R^2; **c**, **d**) values calculated from urine or plasma isotope enrichments. For all panels, *symbols* and *error bars* represent mean and standard error, respectively. In Bonferroni-corrected post-tests; *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$
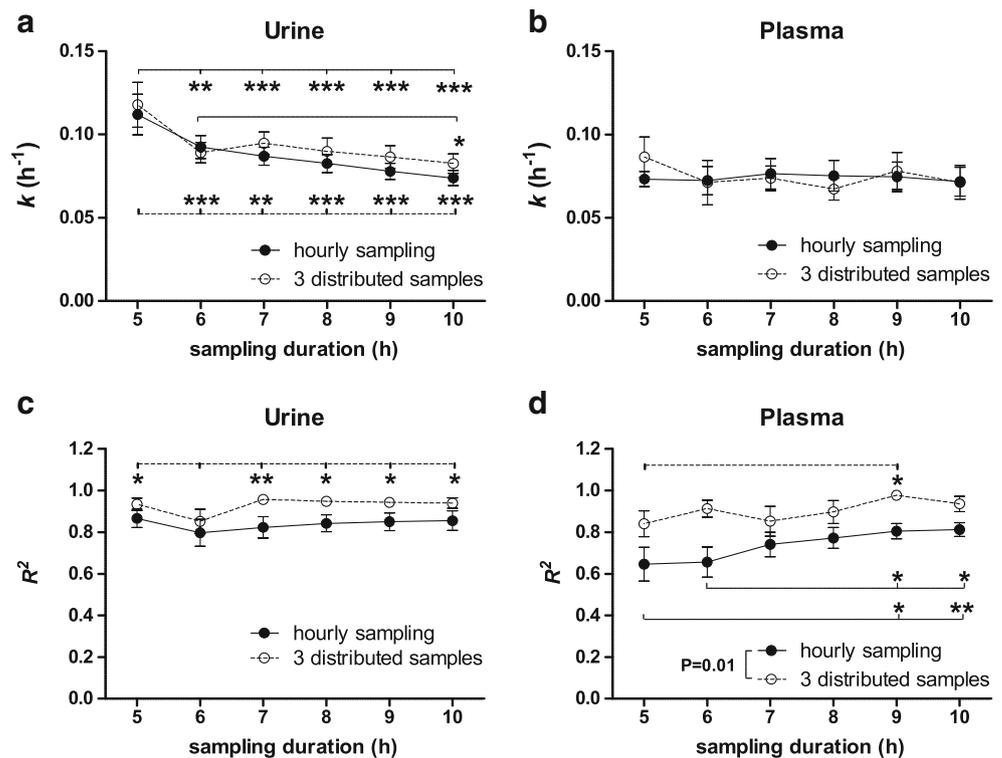
Urinary R^2 values were significantly affected by sampling duration ($P=0.02$ for time main effect), with Bonferroni-corrected post-tests indicating that the R^2 value from distributed sampling over 6 h was slightly but significantly lower than for distributed sampling of all other durations (Fig. 2c). In addition, R^2 values from distributed sampling were marginally higher ($P=0.08$ for main effect of sampling method) than for hourly sampling. Plasma R^2 values were significantly affected by both sampling duration (Fig. 2d, $P=0.003$ for duration main effect) and sampling method (Fig. 2d, $P=0.01$ for main effect of sampling method).

Effect of age on D-3MH enrichment decay A serendipitous natural age break in the subject population allowed comparison
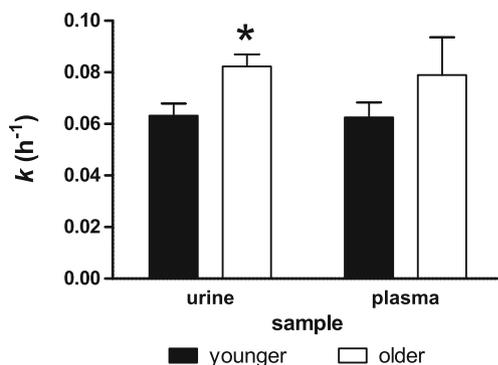

Fig. 3 Decay values (k) calculated from urine or plasma isotope enrichments from younger and older men, *$P=0.02$ vs. younger

between two small groups with significantly different ages (younger, $N=4$, 33 ± 2 years (mean±SD); older, $N=5$, 66 ± 8 years, $P=0.0001$). Urine and plasma decay constants for the two groups from the meatless condition are shown in Fig. 3. Urine decay constants were significantly different between younger and older subjects; plasma decay constants from the two age groups were similar to the urine means but the difference between age groups did not reach statistical significance.

4 Discussion

Pathological loss of skeletal muscle mass contributes to aging- and disease-associated morbidity and mortality [4, 19, 22–27]. However, there is currently no predictive test that can be used to identify individuals, such as recently diagnosed cancer patients, at risk for such losses. This diagnostic void stymies efficient translation of promising proof-of-concept findings from animal studies, which suggest that prevention of skeletal muscle loss associated with cancer may indeed increase survival [31, 32], into the human clinical realm. Here, we revisited the use of 3-methylhistidine as a potential biomarker for susceptibility to skeletal muscle loss in a proof-of-concept study of healthy men. Our results suggest that by employing a tracer-based method with oral dosing, it is possible to obtain an index of myofibrillar protein breakdown from spot urine or plasma samples collected the day after dosing. Further, this approach

obviates the need for 3 days abstention from meat or 24-h urine collection, which are important practical limitations of nontracer-based methods which measure 3MH concentrations.

Although in this proof-of-concept study samples were collected hourly over a 10-h period, such a regimen may prove burdensome in some patients or clinical settings. Accordingly, we examined whether decay values varied significantly over 5–10 h of hourly sampling and whether three samples distributed over the sampling period yielded different decay values than when hourly sampling was conducted. These comparisons suggest that distributed sampling is at least as good as hourly sampling for describing D-3MH decay and that only 5–6 h of distributed sampling yields decay constants that are not significantly different than those obtained over longer durations. Thus, in practice, the test described here can be employed without undue sampling burden.

Due to a serendipitous age break in our study population, we were able to compare two small groups with mean ages differing by a factor of 2. Although tenuous, the decay values of the older individuals were generally higher than in the young (Fig. 3), consistent with the findings from Trappe et al. [48], who reported that 3MH concentrations were significantly higher in the skeletal muscle interstitium of healthy older men as compared to healthy younger men.

Although the small group sizes of the different age groups are clearly a limitation of the current study, these preliminary findings suggest that larger studies of younger and older men are warranted. Likewise, the exclusions of less healthy individuals and females are limitations of the current study that will need to be addressed in future investigations before the utility of 3MH decay rates for determining susceptibility to muscle loss can be fully considered. A final known limitation of the current study is that possible longer-term (>1 day prior to testing) influences of pre-study diet or activity levels could affect 3MH decay rates. A theoretical limitation of the technique presented here, as indeed with all new methodologies, is that it may be supplanted by a superior technique in the future. There is currently great interest in the development of methodologies to diagnose existing or developing pathological loss of skeletal muscle [51–55], although an "ideal" marker has not yet been identified. An approach utilizing complementary methodologies may ultimately prove ideal, as methods to determine muscle mass *per se* are limited by their retrospective nature (i.e., they detect losses after they have occurred), whereas those that measure the dynamic process of loss may respond differentially to different stages of muscle loss.

In summary, the findings from this study suggest that an index of 3MH production can be obtained from an easily administered test involving oral administration of a stable isotope tracer of 3MH followed by three plasma or urine samples collected over 5–6 h the next day. Further, use of this isotopic approach reduces the time during which individuals must abstain from meat from 3 days to at most 1 day (i.e., from the time of tracer dosing until the completion of sample collection the following day). As the feasibility and practicality of such a diagnostic approach have now been established, future prospective studies examining whether the results of this test in fact predict skeletal muscle loss in cancer patients or other at-risk clinical populations are warranted.

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