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ANALYSIS OF 1- AND 3-METHYLHISTIDINES, AROMATIC AND BASIC AMINO ACIDS IN RAT AND HUMAN URINE

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SUMMARY

A procedure based on automated amino acid analysis has been developed to simultaenously quantify 1-methylhistidine (1-MH), 3-methylhistidine (3-MH), tyrosine, phenylalanine, tryptophan, lysine, histidine and arginine levels in human and rat urines. Deproteinized urine samples containing amino acids in the range 1—10 nmol were analyzed using single-column methodology with ninhydrin detection. Standard curves produced correlation coefficients $\geqslant 0.99$ with duplicate analyses agreeing to within ± 1.9%. Quantitative recovery was ensured by using L-α-amino-β-guanidinopropionic acid as an internal standard. Elution was accomplished in less than 90 min at pH 5.7 with sodium citrate buffers at 45°C and 65°C. Since 3-MH in the rat is acetylated at the α-amino group, rat, but not human, urine ultrafiltrates required acid hydrolysis prior to analysis. The utility of the technique of analysis of 1-MH and 3-MH in human urine was demonstrated for an adult male on a meat-free diet for 21 days; urinary excretion rates for 3-MH and 1-MH were determined to be 3.06 ± 0.10 and 0.72 ± 0.07 μmol/kg body mass/day, respectively. The technique was also used to measure the effect of disuse atrophy of rat skeletal muscle which induced a 40—60% increase in 3-MH. The procedure is also highly suited for measurement of urinary aromatic and/or basic amino acids.

INTRODUCTION

Changes in urinary levels of specific amino acids can provide information about metabolism in the intact organism. Wellner and Meister [1] have recently reviewed alterations of amino acid metabolism in man. In 1954, Tallan et al.

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[2] identified 3-methylhistidine (3-MH) as a normal component of human and animal urine. Subsequent experiments demonstrated that residues of 3-MH occupy specific sites in the primary sequences of actin and myosin [3, 4]. Since 3-MH is not reutilized and is quantitatively excreted, urinary 3-MH levels can provide an index of muscle protein turnover [5—7]. Much less is known about the metabolism of 1-methylhistidine (1-MH) although it occurs in the dipeptide, anserine (β-alanyl-L-1-methylhistidine) [8].

One of the reasons that there is a paucity of information on 1-MH is the lack of a convenient and sensitive method for simultaneously determining both 1-MH and 3-MH. These methylated amino acids are often difficult to resolve from other basic amino acids and ammonia. 3-MH has been determined by automated amino acid analysis, by gas chromatography—mass spectrometry (GC—MS), or by high-performance liquid chromatography (HPLC) [9—11]. Small variations in temperature, pH, ionic strength, resin and column dimensions may affect the resolution of these amino acids.

Additionally, sample preparation plays a critical role in the quantitation of amino acids in physiological fluids. Such fluids usually contain soluble proteins which should be removed prior to analysis. A further consideration is that the α-amino groups of 1-MH and 3-MH in the rat are blocked and require acid hydrolysis prior to separation and quantitation with an amine-specific reagent such as ninhydrin.

This paper describes rapid procedures for the preparation and automated amino acid analysis of protein-free urine specimens. We have optimized our conditions for the resolution of 1-MH and 3-MH from other basic amino acids and ammonia. The protocol also allows for the resolution of tyrosine, phenylalanine and tryptophan in less than 40 min.

EXPERIMENTAL

Reagents

Chemicals were obtained from Fisher Scientific (Cincinnati, OH, U.S.A.) or Sigma (St. Louis, MO, U.S.A.). Standards for amino acid analyses were purchased from Calbiochem-Behring (San Diego, CA, U.S.A.) or Beckman (Palo Alto, CA, U.S.A.). Ninhydrin was obtained from Pierce (Rockford, IL, U.S.A.).

Animals

Male Sprague—Dawley rats were purchased from Charles River Breeding Lab. (Boston, MA, U.S.A.) and maintained in metabolism cages on a Purina Rat Chow diet with a 12 h light/12 h dark cycle.

Urine collection

Rat urine was collected under mineral oil, free of fecal contamination, utilizing Hoeltge stainless-steel separatory funnels and was stored at −20°C prior to analysis. Human urine was collected from a 91-kg normal 38-year-old male (RCF) on a meat-free diet for 21 days. At each urine collection the volume was measured and a 10-ml aliquot was saved in a glass vial containing five drops of toluene. Samples were either frozen immediately or stored at 4°C for up to 48 h. The equivalent of a 24-h urine collection was prepared by

taking an aliquot of each individual sample as a percentage of total daily urine volume. Such a procedure permits analysis of the metabolite content at each collection point as well as the total metabolites present in a 24-h collection. Daily urine collection was started at 12.00 midnight.

Preparation of urine ultrafiltrates

Urine (1 ml) was added to a 75 × 12 mm polystyrene tube containing 38 mg of citric acid (1 ml of 0.2 *M* citric acid; lyophilized) such that the final concentration was 0.2 *M* and the pH range was 2—3. Of a 25 m*M* stock of L-α-amino-β-guanidinopropionic acid (AGP) 10 μl were added to each urine sample as an internal standard. Following mixing, the urine sample was transferred to an Amicon (Lexington, MA, U.S.A.) MPS ultrafiltration apparatus. Ultrafiltrates were obtained by centrifugal ultrafiltration (1500—1700 *g* for 15 min) through a 14-mm YMB membrane having a molecular mass cut-off of 10,000 daltons. Aliquots of each sample were analyzed directly on a Dionex D-300 amino acid analyzer (Sunnyvale, CA, U.S.A.) or were hydrolyzed prior to analysis. Control experiments demonstrated the complete retention of ^{14}C-labeled human albumin and that ultrafiltration had no effect on the recovery of any amino acid determined in this study.

Acid hydrolysis of urines

Rat urines require hydrolysis prior to analysis because the α-amino groups of methylhistidines are acetylated or blocked in peptide linkage and will not react with ninhydrin. For hydrolysis, 250-μl aliquots of the ultrafiltrates were transferred to 2-ml glass ampules. An equal volume of concentrated hydrochloric acid was added and the ampules were sealed under vacuum. After hydrolysis at 110°C for 24 h the samples were dried in a desiccator overnight under house vacuum and for an additional two days with a vacuum pump. Samples were dissolved with 250 μl of loading buffer pH 2.2 [composition 0.2 *M* Na^+; 0.5% (v/v) thiodiglycol; 0.1% (w/v) phenol] and were stored frozen in 400-μl polypropylene tubes (Sarstedt, Princeton, NJ, U.S.A.). Prior to analysis, thawed samples were centrifuged 5 min in a Beckman microfuge.

Amino acid analysis

This procedure, which should be routinely applicable to other automated amino acid analysis systems, represents modifications of a protocol developed for Durrum Instrumentation (May, 1977) and provided by Dionex Corporation. Samples (20—40 μl) were analyzed using single-column methodology with sodium citrate eluents and a ninhydrin detection system. The column (17 cm × 4 mm) was packed with Dionex DC-5A cation-exchange resin (6 μm diameter). The three sodium citrate eluents (buffers A, B, and C) at pH 3.25, 5.68, and 5.72 contained 0.5% (v/v) thiodiglycol, 0.1% (w/v) phenol and Na^+ at 0.2 *M*, 0.35 *M* and 1.0 *M*, respectively. Samples were injected onto the column (previously equilibrated with buffer A) and eluted at a flow-rate of 20 ml/h with buffer B for 61 min at 45°C followed by buffer C for 25 min at 65°C. Ninhydrin reagent was pumped at 10 ml/h with the products monitored at 570 and 440 nm. Between each run the column was washed for 5 min with a 0.1 *M* sodium hydroxide solution containing 0.1 *M* sodium chloride and 1 m*M* EDTA.

It is essential that the column be re-equilibrated for 10 min with buffer A prior to loading the next sample.

Sample quantitation

Samples and standards were run in the range of 1—10 nmol of each amino acid. Individual components were quantitated by measurements of peak heights relative to those of standards. AGP served as an internal standard. Standard curves of 1-MH, 3-MH, and AGP produced correlation coefficients $\geqslant 0.99$. Duplicate analyses routinely agreed to within ± 1.9% with a range of 0—5.3%. Control experiments for the ultrafiltration step have been described above.

Statistics

1-MH and 3-MH levels for the rat and human data are calculated as the mean ± S.E.M. Inter-group significance of rat data (see Fig. 5) was evaluated with a two-tailed Student *t*-test for unpaired samples.

RESULTS

In preliminary experiments the effects of ionic strength, pH and temperature on methylhistidine separation by automated ion-exchange chromatography were investigated. The buffer conditions described in Experimental were found to be optimal. In agreement with the work of others [12], we observed that control of column temperature was important for the resolution of methylhistidines. As shown in Fig. 1, the rate of elution of 3-MH increases more with increasing temperature than does that of histidine or 1-MH. In practice, slight adjustments in temperature and/or pH can be made to achieve optimal resolution. A sample at pH 2—3 is loaded onto a column equilibrated with buffer A at pH 3.25. This allows the separation of 1-MH, 3-MH, tyrosine, phenylalanine, tryptophan, lysine, histidine and arginine (Fig. 1). Total analysis time was shortened by increasing the temperature and ionic strength after 1-MH elution. We have also determined that hydroxylysine elutes 4 min before tryptophan, ornithine 1.5 min before lysine, and the dipeptides, anserine and carnosine, 2.5 and 6 min after lysine, respectively. The method is, therefore, suitable for analysis of methylhistidines, basic and aromatic amino acids.

The methylhistidine levels in ultrafiltrates of human urine can be determined directly. However, the majority of the 3-MH present in rat urine is acetylated at the α-amino group and must be hydrolyzed prior to reaction with ninhydrin. To circumvent this problem, protein-free urine samples are usually hydrolyzed in 2—6 *M* hydrochloric acid at 100—110°C for 1—22 h [5, 12—15]. Because of the variable conditions reported in the literature, the effect of hydrolysis time on the recovery of 3-MH from rat urine was investigated. As shown in Fig. 2 the minimal time required for the hydrolysis of the acetyl groups with 6 *M* hydrochloric acid was 2 h with recovery being quantitative through 48 h. It should be noted that we have assumed 100% recovery following 24 h of acid hydrolysis. This is justified because the hydrolysis loss is less than the 2% error associated with replicate analyses. We routinely hydrolyze rat urine samples for 24 h. This ensures determination of both 1-MH and 3-MH because acetylated 3-MH is quantitatively recovered as 3-MH and the dipeptide, anserine, is hy-

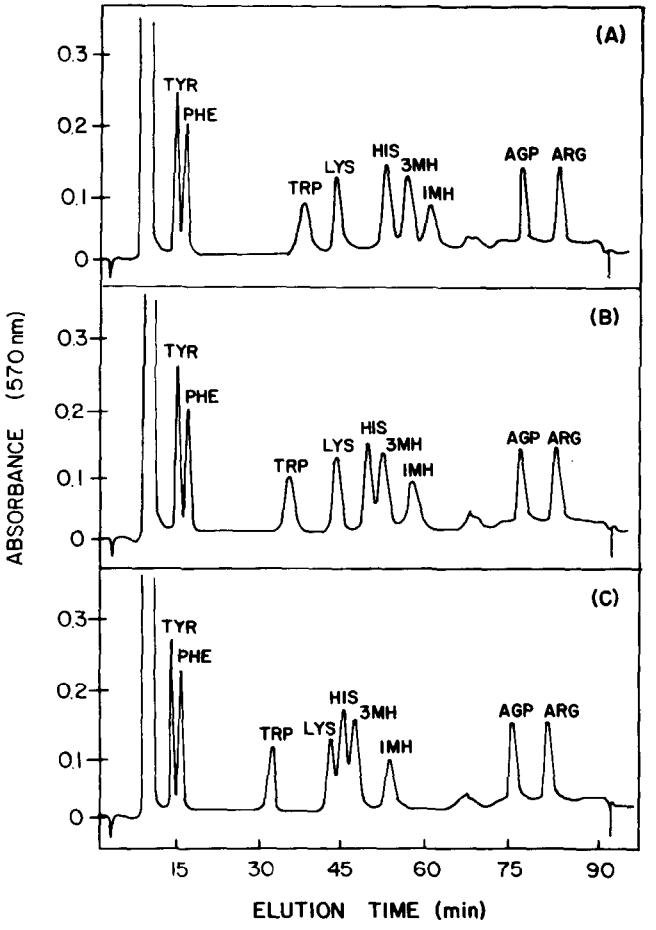

Fig. 1. Effect of temperature on the resolution of 1-MH, 3-MH, basic and aromatic amino acids. Elution conditions are described in Experimental. Panels show the separation of a standard mixture containing 5 nmol of each amino acid. Column temperature for the first 61 min of each analysis was: (A) 45°C; (B) 50°C; (C) 55°C. Peaks: Tyr = tyrosine; Phe = phenylalanine; Trp = tryptophan; Lys = lysine; His = histidine; 3-MH = 3-methylhistidine; 1-MH = 1-methylhistidine; AGP = internal standard; Arg = arginine.

drolyzed to β-alanine and 1-MH. Fig. 3 shows typical analyses of unhydrolyzed human urine and hydrolyzed rat urine. Table I summarizes the effects of acid hydrolysis on the quantitation of 1-MH and 3-MH in human and rat urine. Acid hydrolysis was shown to have no effect on the recovery of 1-MH and 3-MH in human urines confirming that trace amounts of these amino acids occur in the acetylated form or in small peptides [6]. In contrast, 80—90% of 1-MH and 3-MH in rat urine was found to be acetylated or in peptide linkage.

We applied this method to the quantitation of 1-MH and 3-MH in 24-h samples of human urine obtained from a normal human male on a diet free of meat, but containing protein adequate to maintain nitrogen balance. Variations in fluid intake and exercise pattern caused the daily urine output to vary over a four-fold range. The data in Fig. 4 show an inverse relationship between methylhistidine concentration and urine volume. Total daily methylhistidine

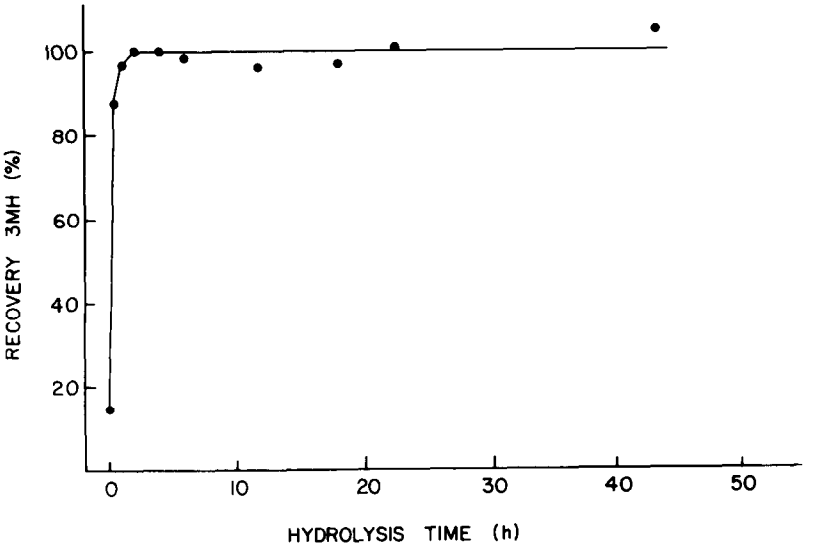

Fig. 2. Effect of hydrolysis time on recovery of 3-MH from rat urine. Ultrafiltrates were hydrolyzed in 6 *M* hydrochloric acid at 110°C in vacuo. Samples were analyzed in duplicate at 0, 0.5, 1, 2, 4, 6, 12, 18, 24 and 48 h.

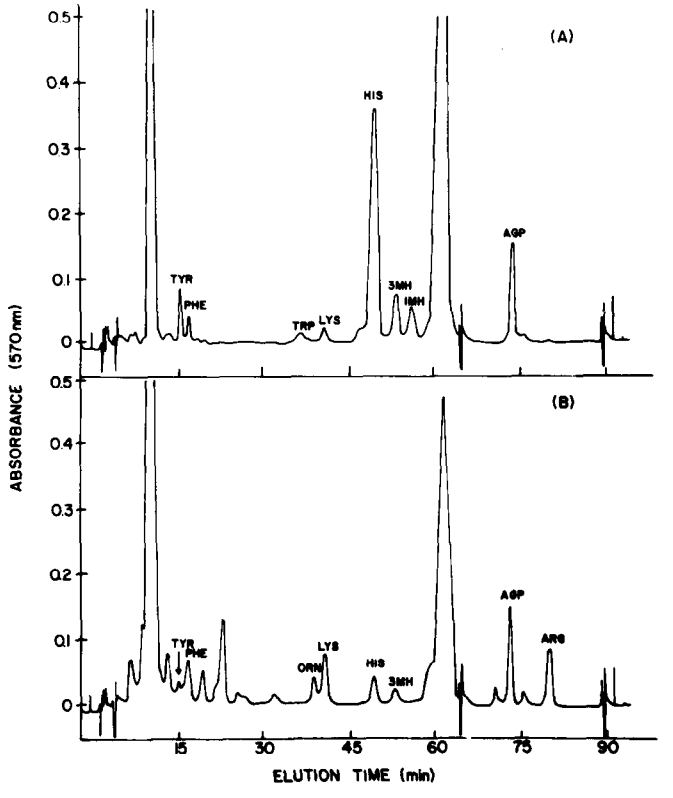

Fig. 3. Typical amino acid analysis of (A) human and (B) rat urine. Ultrafiltrates of urine were prepared and analyzed as described in Experimental. Rat urine but not human urine was hydrolyzed for 24 h prior to analysis. For peak identification see Fig. 1; Orn = ornithine.

TABLE I

EFFECT OF HYDROLYSIS ON DETERMINATION OF 1-MH AND 3-MH

Urine	*n*	3-MH (nmol per 20 μl ± S.E.M.		1-MH (nmol per 20 μl ± S.E.M.	
		No hydrolysis	24-h Hydrolysis	No hydrolysis	24-h Hydrolysis
Human	9	5.00 ± 0.42	5.11 ± 0.45	1.21 ± 0.21	1.07 ± 0.23
Rat	4	0.78 ± 0.13	5.02 ± 0.31	1.36 ± 0.34	9.42 ± 0.81

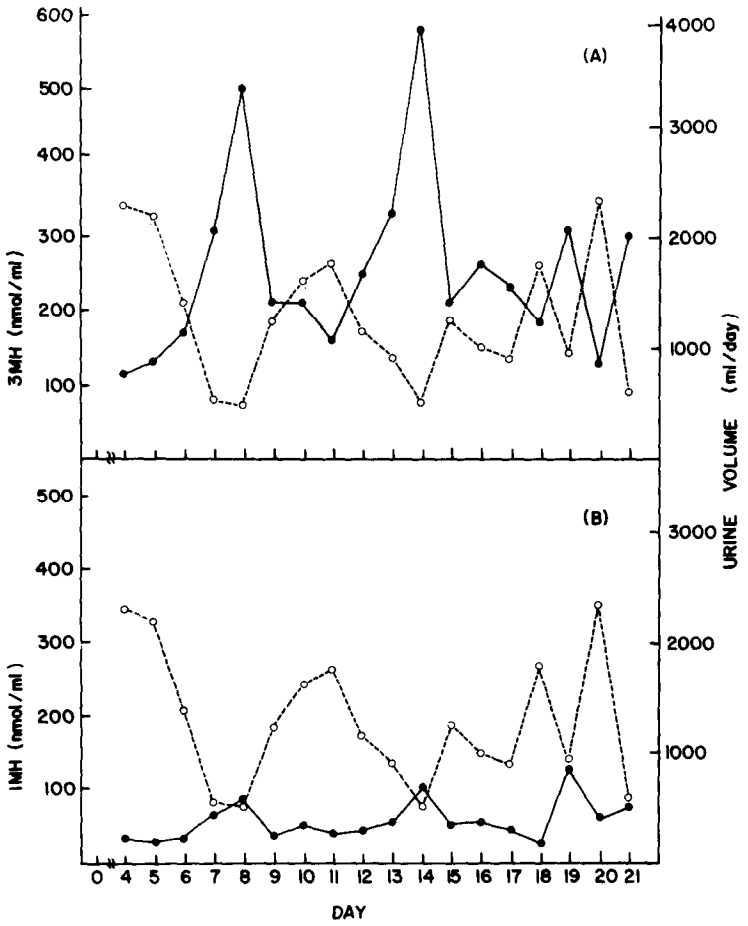

Fig. 4. Daily variations in urine volume (○) and concentration (●) of (A) 3-MH and (B) 1-MH. Urine from an adult human male on a meat-free diet was analyzed as shown in Figs. 1 and 3.

excretion was obtained by multiplying urine volume (ml/day) × MH concentration (nmol/ml). For this 91-kg individual 3-MH and 1-MH excretion averaged 278 ± 9.1 and 66 ± 6.2 μmol/day, respectively. Alternatively, the results for 3-MH and 1-MH can be expressed per kg body mass as 3.06 ± 0.10 and 0.72 ± 0.07 μmol/kg/day, respectively.

The procedure was also utilized for the experiments shown in Fig. 5; the data depict alterations in 3-MH excretion resulting from disuse atrophy of rat hindlimb muscles. Disuse was induced by a whole body suspension technique

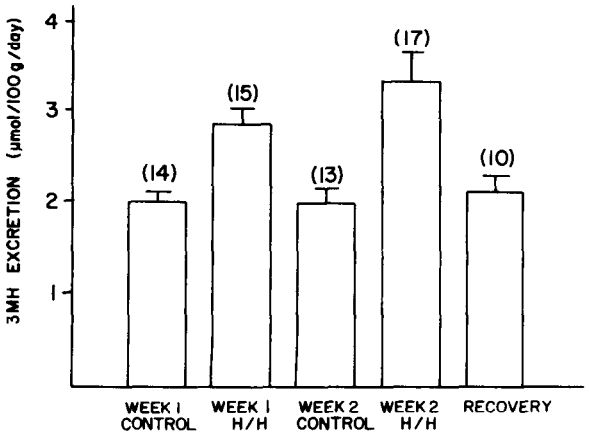

Fig. 5. Effect of hindlimb muscle disuse and recovery on the average daily urinary excretion of 3-MH in the rat. Hindlimb disuse was induced by a hypokinetic/hypodynamic (H/H) suspension as previously described by Musacchia et al. [16]. Some rats were removed from the suspension apparatus after one week and maintained in metabolism cages to study recovery. Ultrafiltrates of urines were hydrolyzed and analyzed as described in Figs. 1—3. Number of rats are indicated in parentheses. The error bars indicate 1 S.E.M.

previously demonstrated to produce hindlimb muscle atrophy through both hypokinesia (reduced limb movement) and hypodynamia (decreased mechanical loading) [16]. Control animals, housed in metabolism cages for up to two weeks, maintained a uniform rate of 3-MH excretion which approximated 2 μmol per 100 g per day. Hindlimb disuse for one and two weeks significantly ($P<0.001$) elevated urinary 3-MH excretion by 40—60%, consistent with the increased protein turnover associated with muscle atrophy. One week of recovery, following removal of rats from the suspension apparatus and return to metabolism cages, was associated with a decrease in 3-MH excretion to values not significantly different from those observed in controls. These results demonstrate that the present technique can be utilized to assess metabolic alterations at the tissue level.

DISCUSSION

This paper describes efforts to develop a rapid, quantitative procedure for the simultaneous analysis of 1-MH and 3-MH in rat and human urine. These metabolites are derived from the catabolism of methylated peptides and proteins [17]. Although there is some controversy [18, 19] it appears that urinary levels of 3-MH can reflect muscle protein turnover when mammals are maintained on a diet lacking in 3-MH for at least four days [5—7].

A variety of methods have been employed for the quantitation of 3-MH in physiological specimens [9—11]. A protein-free sample is usually prepared prior to the actual analysis. Blanchard [20] has reviewed some of the common techniques for sample deproteinization. We have utilized a centrifugal ultrafiltration deproteinization procedure for the preparation of urine samples for methylhistidine analysis. In a single step, each 1-ml sample of urine is adjusted to pH 2—3, mixed with an internal standard and made protein-free. A dozen

human urine samples can be prepared for automated amino acid analysis in 0.5 h.

The procedure also allows for the quantitation of phenylalanine, tyrosine, tryptophan, lysine, histidine, arginine, ornithine, hydroxylysine, anserine and carnosine. Since alterations in the urinary levels of several of these amino acids have been correlated with various disease states this procedure could be helpful in diagnosis and monitoring [1].

Measurements of 3-MH in rat urine are complicated by the increasing amount of acetylation which occurs during development [5]. We found that a minimum of 2 h of hydrolysis in 6 *M* hydrochloric acid at 110°C was required, although recovery was quantitative for all time points up to 48 h. Ammonia does not interfere with the determination of 1-MH by our method although it does in other procedures [17, 21].

Presently, the automated determination of methylhistidines and other amino acids in physiological specimens is accomplished by GC—MS, HPLC or ion-exchange chromatography. GC—MS techniques require sample derivatization prior to analysis and relatively expensive, specialized equipment [10, 22]. Most automated ion-exchange chromatographic procedures for 3-MH determination require 4—6 h per analysis and only provide information on one of the methylhistidines [9, 12, 17, 23]. HPLC methods are often rapid with high sensitivity. Samples can be quantitated by ultraviolet or fluorescent detection following pre-column or post-column derivatization.

However, none of the published methods allow for the simultaneous determination of 1-MH and 3-MH from rat or human urine [11, 24, 25]. Additionally, the resolution of acetylated 1-MH and 3-MH has not been demonstrated. Our procedure, based on high-sensitivity single-column amino acid analysis coupled with deproteinization by centrifugal ultrafiltration, allows for the rapid quantification of 1-MH, 3-MH, basic and aromatic amino acids in human urine. The data (Fig. 4) agree with other reported values [21, 24, 26, 27]. As noted previously, determination of methylhistidine levels in rat urine usually requires hydrolysis. The daily excretion of 3-MH reported here is similar to urine concentrations determined by ion-exchange chromatography based on 4-h analyses [13, 14]. Our procedure may be useful for assessing alterations in muscle metabolism as a consequence of cancer, other muscle wasting diseases and therapy utilizing total parenteral nutrition.

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REFERENCES

1 D. Wellner and A. Meister, Ann. Rev. Biochem., 50 (1981) 911.
2 H.H. Tallan, W.H. Stein and S. Moore, J. Biol. Chem., 206 (1954) 825.
3 M. Elzinga, J.H. Collins, W.M. Kuehl and R.S. Adelstein, Proc. Nat. Acad. Sci. U.S., 70 (1973) 2687.
4 G. Huszar and M. Elzinga, Biochemistry, 10 (1971) 229.
5 V.R. Young, S.D. Alexis, B. Baliga, H.N. Munro and W. Muecke, J. Biol. Chem., 247 (1972) 3592.
6 C.L. Long, L.N. Haverberg, V.R. Young, J.M. Kinney, H.N. Munro and J.W. Geiger, Metabolism, 24 (1975) 929.
7 V.R. Young and H.N. Munro, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 37 (1978) 2291.
8 A. Christman, Anal. Biochem., 39 (1971) 181.
9 L.N. Haverberg, H.N. Munro and V.R. Young, Biochim. Biophys. Acta, 371 (1974) 226.
10 D.E. Matthews, J.B. Starren, A.J. Drexler, D.M. Kipnis and D.M. Bier, Anal. Biochem., 110 (1981) 308.
11 L.C. Ward, M. Miller and S. Hawgood, J. Chromatogr., 223 (1981) 417.
12 M. Elzinga and N. Alonzo, Methods Enzymol., 91 (1983) 8.
13 L.N. Haverberg, L. Deckelbaum, C. Bilmazes, H.N. Munro and V.R. Young, Biochem. J., 152 (1975) 503.
14 F.M. Tomas, H.N. Munro and V.R. Young, Biochem. J., 178 (1978) 139.
15 R.W. Wannemacher, Jr. and R.E. Dinterman, Biochem. J., 190 (1980) 663.
16 X.J. Musacchia, D.R. Deavers, G.A. Meininger and T.P. Davis, J. Appl. Physiol., 48 (1980) 479.
17 W.K. Paik and S. Kim, Adv. Enzymol., 42 (1975) 227.
18 F.J. Ballard and F.M. Tomas, Clin. Sci., 65 (1983) 209.
19 M.J. Rennie and D.J. Millward, Clin. Sci., 65 (1983) 217.
20 J. Blanchard, J. Chromatogr., 226 (1981) 455.
21 M. Neuhäuser and P. Fürst, Anal. Biochem., 92 (1979) 294.
22 L. Cotellessa, F. Marcucci, D. Cani, P. Sfondrini, L. Colombo, E. Mussini and F. Poy, J. Chromatogr., 221 (1980) 149.
23 D.T. DiFerrante, N.Y. Wilson and C.S. Leach, J. Chromatogr., 187 (1980) 271.
24 S.J. Wassner, J.L. Schlitzer and J.B. Li, Anal. Biochem., 104 (1980) 284.
25 W.S. Hancock, D.R.K. Harding and Z.V.L. Friedman, J. Chromatogr., 228 (1982) 273.
26 C. Bilmazes, R. Uauy, L.N. Haverberg, H.N. Munro and V.R. Young, Metabolism, 27 (1978) 525.
27 M. Elia, A. Carter, S. Bacon and R. Smith, Clin. Sci., 59 (1980) 509.