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DETERMINATION OF 6-N-TRIMETHYLLYSINE IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for determination of 6-N-trimethyllysine in urine is described. Trimethyllysine and the chemically analogous 6-N-triethyllysine internal standard were isolated from aqueous samples by microcolumn ion-exclusion chromatography. The specimens were derivatized by reaction with 1-fluoro-2,4-dinitrobenzene and reaction byproducts extracted by organic solvents. The trimethyllysine and internal standard derivatives were separated easily from other sample constituents by reversed-phase paired-ion high-performance liquid chromatography with spectrophotometric detection at 405 nm. Standard curves were linear over a sample concentration range of 10—150 nmol/ml; the detection limit corresponded with 0.1 nmol trimethyllysine injected into the chromatograph. The procedure was used for determination of trimethyllysine in human urine.

INTRODUCTION

The amino acid 6-N-trimethyllysine occurs as a minor constituent of proteins in a variety of organisms [1]. Trimethyllysine has been identified in cytochrome *c* of yeast [2] and wheat germ [3], myosin [4], histone [5], and is present in the plasma and urine of human subjects [6]. Trimethyllysine arises in mammals via post-translation protein-specific methyltransferase mediated reactions in which S-adenosylmethionine functions as the methyl group donor [7].

The metabolism of trimethyllysine following proteolysis has been the subject of recent study. The mitochondrial fatty acyltransferase system cofactor carnitine [3-hydroxy-4-(N,N,N-trimethylammonio)-butanoate] has been shown to be synthesized from lysine in the rat [8, 9], and specifically from 6-N-trimethyllysine in that animal [10]. Several other trimethyllysine metabolites

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have been identified; these include the immediate carnitine precursor 4-(N,N,N-trimethylammonio)-butanoate [10], 5-(N,N,N-trimethylammonio)-pentanoate [10], 3-hydroxy-6-N-trimethyllysine [11—13], 2-N-acetyl-trimethyllysine [12], and 2-oxo-6-(N,N,N-trimethylammonio)-hexanoate [14].

Chromatographic identification and determination of trimethyllysine has been accomplished by paper chromatography [6], paper electrophoresis [15], thin-layer chromatography [16], and column cation-exchange chromatography [5, 17]. A number of separations of the three possible 6-N-methylated lysines from basic amino acids have been achieved by cation-exchange chromatography in amino acid analysis systems [18—24]. Extremely long separation times are required, with trimethyllysine eluting after 9 h and more than 24 h required for complete chromatographic development in the system which best resolves the three 6-N-methylated lysines [23]. These methods provide poor sensitivity owing to the large peak elution volumes obtained after 6—9 h. A more rapid separation requiring only 90 min has been reported recently [25], but 6-N-trimethyllysine is not resolved completely from the other 6-N-methylated lysines by this system.

Our interest in the details of the metabolic disposition of trimethyllysine prompted us to develop a method for its automated determination in large numbers of urine samples. The procedure reported below consists of a rapid and specific isolation of trimethyllysine and an analytically appropriate internal standard from urine specimens by ion-exclusion chromatography, pre-column derivatization by reaction with 1-fluoro-2,4-dinitrobenzene, and chromatographic determination by reversed-phase paired-ion high-performance liquid chromatography (HPLC) with spectrophotometric detection.

EXPERIMENTAL

Equipment

The liquid chromatograph consisted of a Model 6000A solvent delivery system, Model U6K syringe loading injection valve, RCM-100 radial compression module, and Model 440 fixed-wavelength absorbance detector purchased from Waters Assoc. (Milford, MA, U.S.A.). A precolumn constructed of zero dead volume chromatographic fittings (Crawford Fitting Co., Solon, OH, U.S.A.) and packed with Co:Pell ODS reversed-phase pellicular chromatographic medium (Whatman, Clifton, NJ, U.S.A.) preceeded the column compression unit in the eluent stream. The chromatographic separation was accomplished on a 10 × 0.8 cm cartridge of 10 μm nominal particle diameter Radial-Pak C_{18} (Waters), and the detector output signal was recorded by a Linear Instruments (Irvine, CA, U.S.A.) chart recorder. A Waters Model 710A automatic sample injection unit and Hewlett-Packard (Avondale, PA, U.S.A.) Model 3354C chromatographic data system were used during routine determination of trimethyllysine in large numbers of samples. In early stages of the project a Waters μBondapak C_{18} reversed-phase column was employed for separations related to the development of the sample isolation procedure and optimization of the derivatization reaction.

Materials

Trimethyllysine and [Me-^{14}C] trimethyllysine were synthesized and purified as described [10]. Triethyllysine was prepared and purified for use as a chromatographic internal standard by a modification of the same procedure in which iodoethane replaced iodomethane as the alkylating agent. The methylated amino acids 1-methylhistidine, 3-methylhistidine, N^G-methylarginine, N^G,N^G-dimethylarginine, N^G,N'^G-dimethylarginine, N^6-methyllysine and N^6,N^6-dimethyllysine were purchased from Calbiochem-Behring (San Diego, CA, U.S.A.). Aqueous stock solutions of trimethyllysine and triethyllysine were prepared and standardized spectrophotometrically [26]. Aqueous trimethyllysine standard solutions were prepared by serial dilution of the stock solution. A working internal standard solution was prepared at 200 nmol/ml concentration. Dowex 1-X8 (200—400 mesh, Cl^- form) anion-exchange resin was purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.), converted to the OH^- form according to the vendor's instructions, and rinsed with water until the effluent pH was neutral. 1-Fluoro-2,4-dinitrobenzene was purchased from Koch-Light Labs. (distributed by Research Products International, Elk Grove Village, IL, U.S.A.); a 2% (w/v) ethanolic solution was prepared daily for use in the derivatization procedure. 1-Heptanesulfonic acid sodium salt was obtained from Eastman Organic Chemicals (Rochester, NY, U.S.A.). Acetonitrile (non-spectro grade) was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Distilled water was prepared for use as a chromatographic eluent constituent by passage through the mixed bed ion-exchange and activated carbon columns of a Milli-Q water purification unit (Millipore, Bedford, MA, U.S.A.).

Sample preparation

In a plastic microcentrifuge tube were combined 500 μl of aqueous standard solution or urine specimen and 200 μl of the triethyllysine internal standard solution. The tube was vortexed briefly, and 500 μl of the contents applied to a 7 × 0.5 cm water washed column of Dowex 1-X8 (200—400 mesh, OH^- form) anion-exchange resin contained by a pasteur pipette. Excluded species were eluted from the column by 2 ml of distilled water. The effluent was collected in 13 × 100 mm disposable glass test tubes which subsequently were transferred to a 50°C water bath and their contents brought to dryness by a gentle stream of oil-free compressed air. Samples so prepared may be stored in a refrigerator prior to derivatization and chromatography.

Derivatization

The dry residue from the isolation procedure was reconstituted in 150 μl of distilled water. To this were added in sequence 250 μl of 1 *M* sodium bicarbonate and 1 ml of the 1-fluoro-2,4-dinitrobenzene reagent solution. The tubes were vortexed, covered by a marble, and placed within a shaking water bath at 37°C for 1 h. At the conclusion of the procedure, the tubes were transferred to a 50°C water bath and their contents evaporated to dryness under a compressed air stream.

To the dried residue were added 4 ml of diethyl ether and the tubes vortexed for 10 sec. The ether was removed by vacuum aspiration, with care taken to

avoid removal of any insoluble material. This extraction step was repeated. The solid residues were then reconstituted in 300 μl 1.4 *M* hydrochloric acid by vortexing for 10 sec. Diethylether (4 ml) was added, the tubes vortexed, and the ether layer removed. Then 2 ml of *n*-butyl acetate were added, the tubes vortexed, and phase separation facilitated by centrifugation in a table top unit for 5 min at 1250 *g*. After aspiration of the organic layer, the tubes were covered and wrapped with aluminum foil for protection against light. Samples were stored in a freezer at −20°C prior to chromatography.

Chromatographic conditions

The chromatographic mobile phase was 0.01 *M* sodium heptanesulfonate in acetonitrile—water (50:50). In 500 ml of purified water were dissolved 2.20 g (0.01 mol) 1-heptanesulfonic acid sodium salt and the solution filtered through a 0.2-μm pore diameter cellulose nitrate membrane. To this filtered solution were added 500 ml filtered acetonitrile with thorough magnetic stirring. The eluent was pumped at 2 ml/min; absorbance was monitored at 405 nm. The sample injection volume was 50 μl in all cases.

Quantitation

Standard curves of trimethyllysine:triethyllysine peak height or area ratios vs. concentration were established daily over a trimethyllysine standard solution concentration range of 10—150 nmol/ml. Trimethyllysine concentrations in experimental samples were interpolated from the least squares regression line through the standard data points. All standard and experimental samples were analyzed in duplicate.

RESULTS AND DISCUSSION

It was our purpose in this work to develop a simple and specific method for determination of urinary 6-N-trimethyllysine. We discovered immediately that accurate measurement of the small urinary quantities of trimethyllysine was complicated by comparatively enormous accompanying quantities of amino acids of all acidity categories. The necessary degree of sample simplification was achieved by ion-exclusion chromatography on small columns of the strong anion-exchange resin Dowex 1-X8 (OH^- form), by which anions are quantitatively adsorbed. Cations and polar species without net charge at the high effective pH of the resin surface are unretained and eluted by distilled water. Recovery of trimethyllysine was investigated by application of [Me-^{14}C] trimethyllysine to the sample preparation columns; 96% of the applied radioactivity was recovered in the 2 ml of distilled water used for elution of excluded species.

A series of experiments intended to verify the removal of nonquaternary N-methylated amino acids by the Dowex-1 column were performed. Aqueous solutions of 1-methylhistidine, 3-methylhistidine, N^G-methylarginine, N^G,N^G-dimethylarginine, N^G,N'^G-dimethylarginine, N^6-methyllysine and N^6,N^6-dimethyllysine were prepared. Each was subjected to the derivatization procedure and the k' value for each derivative determined on the μBondapak C_{18} column used in early stages of the project. Only the N^G-methylarginine 2,4-

dinitrophenylamino derivative was found to be retained similarly to the trimethyllysine derivative by this column; the two peaks eventually were resolved with considerable experimental difficulty. One micromole of each amino acid was then applied to the Dowex-1 (OH^- form) resin sample preparation columns, the columns eluted with distilled water, and the collected effluent derivatized and chromatographed. The chromatograms obtained in experiments with all of the tested compounds except N^G-methylarginine were indistinguishable from those obtained upon derivatization of an identically treated sample of water. Some N^G-methylarginine was found to be eluted from the Dowex-1 column by distilled water, but none of this compound was detected in the effluent of sample preparation columns eluted with dilute ammonium hydroxide at pH 12.5.

[Me-^{14}C]Trimethyllysine was subjected to a series of derivatization reaction optimization experiments in which the labelled precursor and its 2,4-dinitrophenylamino derivative were separated by HPLC. The column effluent was collected in 0.5-ml fractions and the radioactivity determined in each fraction by liquid scintillation counting. Two radioactive peaks were detected. The first was barely retained by the reversed-phase column and co-chromatographed with injected [Me-^{14}C]trimethyllysine. The second peak of radioactivity appeared coincidentally with a single spectrophotometrically detectable peak, which in turn was shown to vary in size directly with sample trimethyllysine concentration. The reaction was found to proceed to 85% (± 3%, n=5) completion with manageable chromophoric byproduct formation under the reaction conditions described.

A series of extraction steps were developed for removal of residual reagent and chromophoric side reaction products. Recovery of [Me-^{14}C]trimethyllysine was verified: 3% of the radioactivity present in samples after the ion-exclusion sample preparation step was found in the organic solvents employed for the extractions, while the remaining 97% was found in the final aqueous acid specimen ready for chromatography. Since 96% of applied [Me-^{14}C]trimethyllysine was recovered from the sample preparation columns, the recovery of trimethyllysine throughout the preparation sequence prior to chromatography was 93%. The photosensitivity of amino acid 2,4-dinitrophenylamino derivatives has been described [27]. It was found that [Me-^{14}C]trimethyllysine 2,4-dinitrophenylamino derivatives did undergo slow decomposition to another chromatographically distinguishable labelled species upon either exposure to light or storage at room temperature. Specimens prepared for chromatography therefore were protected against exposure to light and stored overnight at low temperature prior to analysis.

Application of the developed sample isolation and derivatization procedure to urine specimens revealed the presence of an interfering endogenous sample constituent which was both unretained by the ion-exclusion columns used for sample preparation and reactive toward 1-fluoro-2,4-dinitrobenzene. Systematic manipulation of the eluent counter-ion concentration, pH, and organic modifier concentration and identity did not adequately resolve the trimethyllysine derivative and the sample contaminant on the μBondapak C_{18} column initially used in this work. Further experimentation with higher and lower chain length alkanesulfonate counter-ions also did not provide the required

selectivity. The necessary separation was achieved on the highly retentive reversed-phase medium Radial-Pak C_{18}, upon which all urine specimens were then chromatographed during the course of this study.

Fig. 1 is a chromatogram of an aqueous blank carried through the analytical scheme. A chromatogram of a processed aqueous trimethyllysine standard solution at 50 nmol/ml which included triethyllysine internal standard is shown in Fig. 2. Fig. 3 is a chromatogram of a derivatized urine specimen containing 21 nmol/ml trimethyllysine. Although the methylhistidines, methyllysines, and methylarginines (other than N^G-methylarginine) tested were retained during the ion-exclusion chromatographic sample preparation step, their 2,4-dinitrophenylamino derivatives were shown to be separated from those of trimethyllysine and the internal standard under these chromatographic conditions. The trimethyllysine metabolite 3-hydroxy-6-N-trimethyllysine was not retained by the anion-exchange resin and therefore in principle could be determined if present in samples at sufficient concentration. The time required for the analysis may be reduced by operating the instrument at solvent flow-rates as high as 4 ml/min without unacceptable loss of resolution of the trimethyllysine derivative and the preceeding more polar endogenous sample constituent.

Standard curves of trimethyllysine:triethyllysine peak height ratios vs. sample trimethyllysine concentration were found to be linear over a sample concentration range of 10—150 nmol/ml trimethyllysine (r^2=0.99) and to pass through the graphic origin. The slopes of 50 standard curves varied with a relative standard deviation of 6% during the one-year course of the study. The detection limit at a signal-to-noise ratio of 5:1 was found to correspond

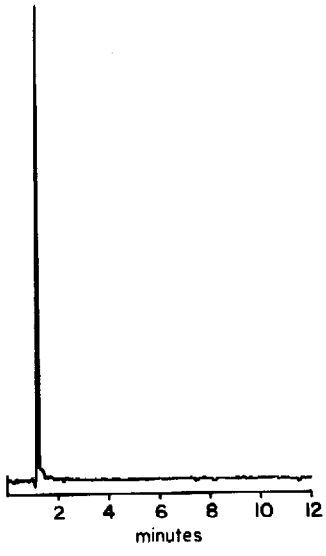

Fig. 1. Chromatogram obtained after complete preparation and derivatization of an aliquot of distilled water according to the described procedure. The column was a 10 × 0.8 cm cartridge of 10-μm nominal particle diameter Radial-Pak C_{18} (Waters Assoc.). The chromatographic eluent was $1 \cdot 10^{-2}$ *M* 1-heptanesulfonic acid sodium salt in acetonitrile—water (50:50) and was pumped at 2.0 ml/min. The spectrophotometric detector was operated at 405 nm. The full scale of the ordinate is 0.02 absorbance units.

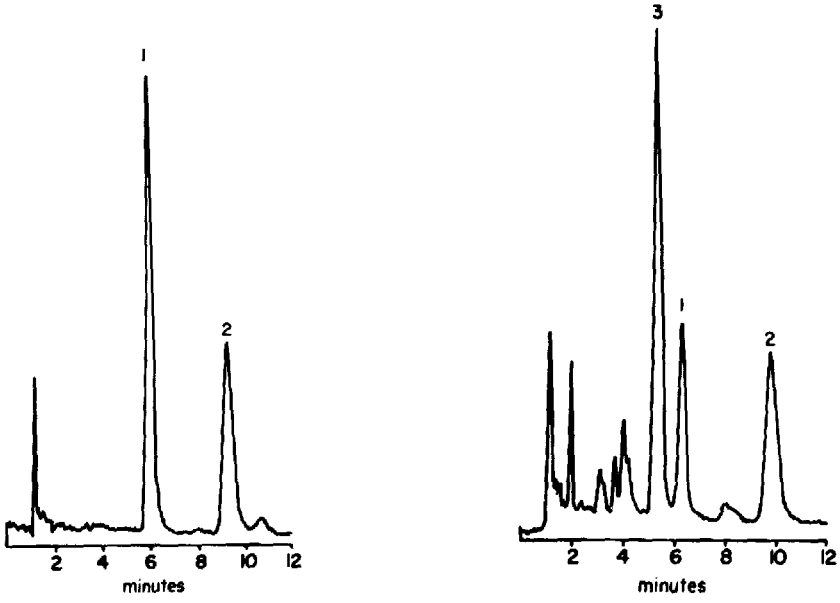

Fig. 2. Chromatogram of a derivatized aqueous standard solution of trimethyllysine at 50 nmol/ml which included triethyllysine the internal standard. The chromatographic conditions were as described in Fig. 1. Peaks: 1 = trimethyllysine 2,4-dinitrophenylamino derivative; 2 = triethyllysine 2,4-dinitrophenylamino derivative.

Fig. 3. Chromatogram of a prepared and derivatized urine specimen containing 21 nmol/ml trimethyllysine. The chromatographic conditions were as described in Fig. 1. Peaks: 1 = trimethyllysine 2,4-dinitrophenylamino derivative; 2 = triethyllysine 2,4-dinitrophenylamino derivative, internal standard; 3 = urine sample constituent unretained by ion-exclusion sample preparation columns.

with 0.1 nmol of trimethyllysine injected into the chromatograph. Mean deviations for duplicate sample determinations were typically less than 5%. A dependence of the absolute retention of trimethyllysine and the internal standard upon injection volume was noted. When the injection volume was fixed at 50 μl, absolute retention of the derivative peaks varied by less than 1% from injection to injection. More than 2500 trimethyllysine determinations were performed in the course of this investigation; throughout the project, the chromatographic performance of the Radial-Pak C_{18} cartridge was satisfactory.

Urinary excretion of trimethyllysine by human subjects

A healthy female volunteer of normal body weight ingested Ensure brand isocaloric liquid diet for two days and then fasted for three days. Urinary trimethyllysine excretion was found to be 36.4 μmol per 24 h on the day prior to fasting. The values obtained during the fasting period were 38.4, 39.2, and 37.5 μmol per 24 h on the first, second, and third days of fasting, respectively. Although these values fall below the ranges reported by Kakimoto and Akazawa [6], Löwer et al. [28] and Lou and Siena [29], our test subject consumed a diet (Ensure) which has a very low content of trimethyllysine. Löwer et al. [28] reported a mean daily urinary excretion of 64 μmol tri-

methyllysine with wide variation among individuals in both total excretion of methylated lysines and in trimethyllysine as a fraction of total excreted methylated lysines. Kakimoto and Akazawa [6] reported no change in urinary trimethyllysine excretion by human subjects ingesting a protein-free diet for 30 h. Our own comparison of trimethyllysine excretion by normal-weight and obese subjects will be reported elsewhere [30].

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