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DETERMINATION OF FREE TRIMETHYLLYSINE IN PLASMA AND TISSUE SPECIMENS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the determination of 6-N-trimethyllysine in tissues and plasma is described. Trimethyllysine and the chemically analogous 6-N-triethyllysine (internal standard) were isolated from acid-soluble fractions of tissue homogenates or plasma by combined ion-exchange—ion-exclusion chromatography. Trimethyllysine and triethyllysine were separated from other sample constituents by reversed-phase ion-pair high-performance liquid chromatography, derivatized post-column by reaction with *o*-phthalicdicarboxaldehyde and 2-mercaptoethanol, and detected fluorometrically. Standard curves were linear over a sample concentration range of 0.5—4 nmol/ml. The detection limit corresponded with 25 pmol trimethyllysine injected into the chromatograph. The procedure was used for the determination of trimethyllysine in plasma, liver, kidney, and mixed skeletal muscle of rat.

INTRODUCTION

The amino acid 6-N-trimethyllysine occurs in numerous proteins, including histones [1] and myosin [2]. Trimethyllysine is formed in mammals by the action of protein methylase III and S-adenosylmethionine upon selected lysine residues in specific proteins [3]. Once released from protein during proteolysis, trimethyllysine is known to be metabolized via the carnitine biosynthetic pathway [4—6] and to be excreted in the urine [7]. Carnitine [3-hydroxy-4-(N,N,N-trimethylammonio) butanoate] is an essential cofactor in mitochondrial long-chain fatty acid oxidation. The control of the fate of trimethyllysine is not understood, in large part, owing to the lack of sensitive analytical methods. The determination of free trimethyllysine is important not only in

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understanding trimethyllysine metabolism but in the delineation of the regulation of carnitine biosynthesis.

Trimethyllysine has been determined by a number of lengthy procedures of varying sensitivity incorporating ion-exchange chromatography and traditional amino acid analysis [8—14]. We have previously developed a sensitive method for the determination of urinary trimethyllysine concentrations by high-performance liquid chromatography (HPLC) [15]. However, this method does not provide a limit of sensitivity adequate for practical measurement of free trimethyllysine in plasma or tissue specimens.

The reaction of primary amines with *o*-phthalicdicarboxaldehyde and alkanethiols to produce intensely fluorophoric 1-alkylthio-2-alkyl-isoindoles [16] has been applied in the development of very sensitive methods for determination of amino acids at detection limits far lower than possible when ninhydrin or fluorescamine are used as derivatizing agents [17, 18]. We have applied this derivatization process in the development of a method for the determination of free trimethyllysine concentrations in plasma and tissue specimens. In this procedure, trimethyllysine and a chemically analogous internal standard are isolated from plasma and tissue homogenates by protein precipitation and combined ion-exchange—ion-exclusion chromatography of the protein-free supernatant. Determination is accomplished by reversed-phase ion-pair HPLC, postseparation column derivatization by reaction with *o*-phthalicdicarboxaldehyde and 2-mercaptoethanol, and fluorometric detection.

EXPERIMENTAL

Equipment

The liquid chromatograph consisted of a single Model M6000A pump, a Model U6K syringe loading injection valve, and an RCM-100 radial compression module purchased from Waters Assoc. (Milford, MA, U.S.A.). A precolumn constructed of zero dead volume chromatographic fittings (Crawford Fitting, Solon, OH, U.S.A.) and packed with Co:Pell ODS reversed-phase pellicular chromatographic medium (Whatman, Clifton, NJ, U.S.A.) preceded the column compression unit in the eluent stream. The chromatographic separation was accomplished on a 10 × 0.5 cm cartridge of 10 μm nominal particle diameter Radial-Pack C_{18} (Waters). The postcolumn derivatization reagent solution was delivered by a Milton-Roy minipump (Glenco Scientific, Houston, TX, U.S.A.), and introduced within the eluent stream through a zero dead volume T fitting (Waters). A 50 × 0.023 cm coil of steel tubing provided a short reaction time delay prior to detection. A Kratos-Schoeffel Instruments (Westwood, NJ, U.S.A.) Model SF-970 fluorescence detector was used for eluent fluorescence measurements. The detector output signal was recorded by a Linear Instruments (Irvine, CA, U.S.A.) Model 291 chart recorder. Peak area integration and height measurements were performed by a Hewlett-Packard (Avondale, PA, U.S.A.) Model 3354 laboratory automation system. Liquid scintillation counting was performed with a Packard Instruments (Downers Grove, IL, U.S.A.) PRIAS liquid scintillation counter. A Brinkman (Des

Plaines, IL, U.S.A.) Polytron tissue homogenizer, DuPont/Sorvall (Newtown, CT, U.S.A.) table-top centrifuge and a Buchler Instruments Evapomix evaporator (distributed by Fisher Scientific, Cleveland, OH, U.S.A.) were used during sample preparation.

Materials

MCB OmniSolv acetonitrile (non-UV grade) was purchased from Curtin Matheson Scientific (Cleveland, OH, U.S.A.). Sodium dodecyl sulfate (electrophoresis grade) was obtained from Gallard-Schlesinger (Carle Place, NY, U.S.A.). Hydrochloric acid, ammonium hydroxide, sodium hydroxide, and sodium dihydrogen phosphate were obtained from Fisher Scientific. Boric acid, *o*-phthalicdicarboxaldehyde, and the polyether surfactant Brij-35 were obtained from Aldrich (Milwaukee, WI, U.S.A.). The ion-exchange resins Dowex 50W-X8 (200—400 mesh, H^+) and Dowex 1-X8 (200—400 mesh, Cl^-) were purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). These resins were washed with water and converted to their ammonium and hydroxide forms, respectively, according to the vendor's instructions. Water was prepared for use as a chromatographic eluent constituent by passage through a Milli-Q water purification unit (Millipore, Bedford, MA, U.S.A.).

Trimethyllysine was prepared as described previously [4]. 6-N-Triethyllysine was prepared for use as an analytical internal standard by a modification of the same procedure in which iodoethane replaced iodomethane as the alkylating agent. 3-Hydroxy-6-N-trimethyllysine was prepared in our laboratory by a procedure which will be reported elsewhere. [^{14}C-Methyl]trimethyllysine was prepared as reported [4]. Bovine serum albumin, 1-N-methylhistidine and 3-N-methylhistidine were obtained from Sigma (St. Louis, MO, U.S.A.). N^G-Methylarginine and N^G,N'^G-dimethylarginine were purchased from Calbiochem-Behring (San Diego, CA, U.S.A.). 6-N-Methyllysine and 6-N-dimethyllysine were purchased from Chemical Dynamics (South Plainfield, NJ, U.S.A.).

Aqueous stock solutions of trimethyllysine and triethyllysine were prepared and standardized spectrophotometrically [19]. Standard solutions of trimethyllysine for use in both plasma and tissue determinations were prepared by serial dilution of the stock solutions with a 2% (w/v) aqueous solution of bovine serum albumin.

Sample preparation

Plasma specimens. Whole blood was collected in heparinized tubes, chilled on ice and centrifuged at 1500 *g* for 10 min. A 2-ml volume of chilled plasma or standard solution was combined with 0.5 ml 12% perchloric acid and 350 μl of triethyllysine internal standard working solution in a 16.8 × 95 mm disposable polypropylene test tube. The tubes were allowed to stand on ice for 1 h and then centrifuged at 1500 *g* for 10 min. The supernatant was transferred to another 16.8 × 95 mm polypropylene test tube containing 0.5 ml of cold 2 *M* potassium bicarbonate, vortexed, placed on ice for 1 h, and centrifuged at 1500 *g* for 10 min. Volumes of all solutions used in sample preparation were adjusted in direct proportion to sample size when less than 2 ml of plasma were harvested from the blood specimens.

Columns fashioned from 5-ml disposable (10 × 116 mm) polypropylene pipette tips (Kew Scientific, Columbus, OH, U.S.A.) were filled in sequence with 2.5 ml Dowex 1-X8 (OH^-) and 2.5 ml Dowex 50W-X8 (NH_4^+) ion-exchange resins; the Dowex 1-X8 layer was allowed to settle completely before addition of the Dowex 50W cation exchanger. The entire sample supernatant was applied to the column. Excluded and weakly retained species were eluted with 6 ml of 1 *M* ammonium hydroxide. Trimethyllysine and the internal standard then were eluted with 3 ml of 1 *M* ammonium hydroxide and 5 ml of deionized water. The combined 8 ml of column effluent were evaporated to dryness under vacuum in a Buchler Evapomix (water bath at 38° C) and reconstituted in 250 μl of 10^{-3} *M* hydrochloric acid before injection into the liquid chromatograph.

Tissue specimens. To 1 g of rat tissue (liver, kidney, heart, or mixed skeletal muscle) were added 3 ml of ice cold 6% perchloric acid. This mixture was homogenized by the Brinkman Polytron homogenizer operated at setting 6 for 40 sec; 2 ml of the suspension were combined with 350 μl of the triethyllysine internal standard working solution in a 16.8 × 95 mm disposable polypropylene test tube. The tubes were placed on ice for 1 h, centrifuged for 10 min at 1500 *g*, and the supernatant was transferred to another 16.8 × 95 mm polypropylene test tube containing 0.5 ml of 2 *M* potassium bicarbonate. This in turn was vortexed, placed on ice for 1 h, and centrifuged as above. The supernatant liquid was decanted from the potassium perchlorate precipitate and subjected to ion-exchange–ion-exclusion chromatography exactly as were the plasma specimens. The combined effluent fractions were evaporated to dryness under vacuum in a Buchler Evapomix, with the water bath at 38° C. The sample was reconstituted in 250 μl 10^{-3} *M* hydrochloric acid for injection into the chromatograph.

Chromatography

The chromatographic eluent was 30%, v/v acetonitrile in water containing $5 \cdot 10^{-2}$ *M* sodium dodecyl sulfate and $5 \cdot 10^{-2}$ *M* sodium dihydrogen phosphate. To 700 ml of water were added 6.9 g of sodium dihydrogen phosphate and 14.4 g of sodium dodecyl sulfate. The solution was adjusted to pH 2.75 with concentrated sulfuric acid, filtered through a 0.45-μm nominal pore diameter cellulose acetate membrane, and 300 ml of filtered acetonitrile were added with thorough magnetic stirring. The derivatization reagent solution was prepared by dissolving 300 mg of *o*-phthalicdicarboxaldehyde and 500 μl of 2-mercaptoethanol in 10 ml of a 3%, v/v solution of Brij-35 in 95% ethanol and adding this to 500 ml of a 0.5 *M* sodium borate solution of pH 10.4. The eluent and derivatization reagent solutions were delivered at flow-rates of 3.0 ml/min and 1.2 ml/min, respectively. The fluorometric detector was operated at an excitation wavelength of 229 nm with an emission filter cutoff wavelength of 418 nm.

Quantitation

Standard curves of trimethyllysine:triethyllysine peak height or area ratios versus concentration were established daily over a trimethyllysine standard

solution concentration range of 0.5 to 4.0 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

This study was designed for the development of a simple and selective method for the determination of free 6-N-trimethyllysine in plasma and tissue specimens. Sample protein was removed effectively by perchloric acid precipitation. Excess perchlorate was precipitated as its potassium salt during the subsequent sample neutralization with potassium bicarbonate. In a previous study [15], we removed large quantities of interfering acidic and neutral amino acids from urine samples by ion-exclusion chromatography over small columns of Dowex 1-X8 (OH^-) anion-exchange resin prior to derivatization of quaternary ammonio acids and unretained basic amino acids. However, the greater sensitivity of the fluorometric detector revealed that small but troublesome quantities of other primary amines were isolated with trimethyllysine and the internal standard.

The necessary degree of sample simplification was achieved with a larger column containing cation-exchange and anion-exchange resins in serial combination. Elution of this column with 1 *M* ammonium hydroxide resulted in the expected exclusion of basic amino acids and quaternary ammonio acids by the anion-exchange resin, and their partial resolution by cation-exchange chromatography. Recovery of applied [^{14}C-methyl] trimethyllysine from this column was found to be 97% (± 2.2%; $n = 3$).

The reversed-phase, ion-pair separation mechanism can provide excellent chromatographic selectivity for quaternary ammonio acids. In our experience, such separation systems have shown long-term stability and unusual tolerance for sample contaminants. We experimented with several alkylsulfate and alkylsulfonate ion-pairing agents. Sodium dodecyl sulfate of electrophoresis grade was found to provide both greatest chromatographic efficiency for the separation of trimethyllysine from other sample constituents and least mobile phase background fluorescence. Retention of trimethyllysine and triethyllysine was found to vary as expected with changes in the acetonitrile concentration of the mobile phase. Tailing of chromatographic peaks was reduced by inclusion of $5 \cdot 10^{-2}$ *M* sodium dihydrogen phosphate in the aqueous portion of the eluent. The absolute retention of trimethyllysine and the internal standard was found to be sensitive to small alterations in eluent pH from the selected value of 2.75. Recovery of injected [^{14}C-methyl] trimethyllysine through the injector, precolumn, and separation column of the liquid chromatograph was 97% (± 2.7%, $n = 3$). The detection limit corresponded with 25 pmol of trimethyllysine injected into the chromatograph.

The retention of N-methylated amino acids by the ion-exchange—ion-exclusion sample preparation column was investigated. Aqueous solutions of 3-hydroxytrimethyllysine, triethyllysine, 6-N-mono-, di-, and trimethyllysine, 3-N- and 1-N-methylhistidine, and N^G-methyl- and N^G,N'^G-dimethylarginine

TABLE I

RETENTION CHARACTERISTICS OF VARIOUS N-METHYLATED AMINO ACIDS

A 2-nmol amount of each amino acid was applied to and eluted from the sample preparation column. The column effluent was evaporated to dryness and reconstituted in 250 μl of 10^{-3} *M* hydrochloric acid. A second standard portion of 2 nmol of the same amino acid was similarly dried and reconstituted in a second test tube. In succession, a 30-μl aliquot of each sample was injected into the liquid chromatograph. Fractional elution of each amino acid is expressed as the ratio of its chromatographic peak height in the first sample to that in the second, times 100%. These experiments were performed in duplicate.

Amino acid	Percent elution through double resin column	k' (HPLC)
1-N-Methylhistidine	ND*	2.30
3-N-Methylhistidine	ND	2.86
3-Hydroxy-6-N-trimethyllysine	100	3.30
6-N-Methyllysine	ND	4.33
6-N-Dimethyllysine	ND	4.77
6-N-Trimethyllysine	100	5.13
N^G-Methylarginine	30	7.48
N^G,N'^G-Dimethylarginine	58	7.56
6-N-Triethyllysine	100	11.11

*ND = not detected.

were prepared at a 20 nmol/ml concentration. A 2-nmol amount of a single amino acid was applied to the sample preparation column and eluted by the usual series of eluents. Trimethyllsyine, triethyllysine, and 3-hydroxytrimethyllysine were unretained by the sample preparation column, while about 30% of the applied N^G-methylarginine and 60% of the N^G,N'^G-dimethylarginine were eluted. The methylhistidines and the other methyllysines were completely retained by the sample preparation column. These data are summarized in Table I.

The N-methylated amino acids were chromatographed individiually for comparison of their HPLC retention with that of trimethyllysine and triethyllysine. 3-Hydroxytrimethyllysine and the methylhistidines all were retained less strongly than trimethyllysine. The methylarginines eluted between trimethyllysine and triethyllysine, completely resolved from both compounds. 6-N-Methyllysine and 6-N-dimethyllysine were retained less strongly than trimethyllysine. However, 6-N-dimethyllysine was unresolved from trimethyllysine. As seen in Table I, this presented no problem, as 6-N-dimethyllysine was completely retained by the sample preparation column.

Standard curves of trimethyllysine:triethyllysine peak height ratios versus sample trimethyllysine concentration were found to be linear over a sample concentration of 0.5—4.0 nmol/ml trimethyllysine (r^2 = 0.98) with a slightly positive *Y* intercept of 0.07. This intercept value was less than 22% of the peak height ratio obtained for the standard solution containing the smallest trimethyllysine concentration used for standard curve definition. Slopes of five replicate standard curves established on successive days had a relative standard

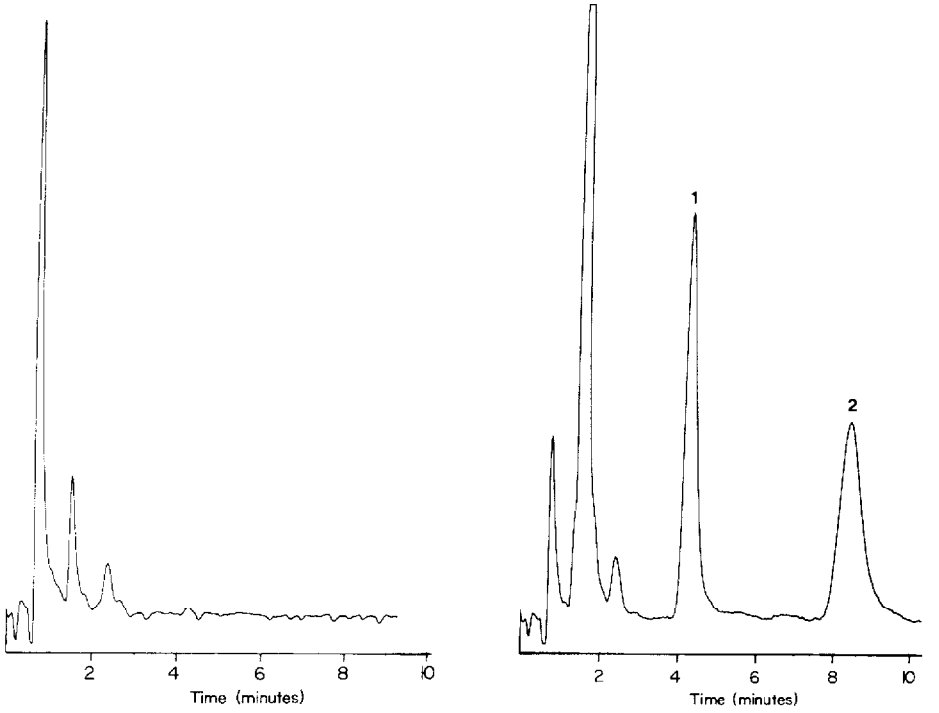

Fig. 1. Chromatogram obtained after complete preparation of an aliquot of 2% (w/v) bovine serum albumin (BSA) according to the described procedure. The column was a 10 × 0.5 cm cartridge of 10-μm nominal particle diameter Radial-Pak C_{18} (Waters). The chromatographic eluent was 30%, v/v acetonitrile in water containing $5 \cdot 10^{-2}$ *M* sodium dodecyl sulfate and $5 \cdot 10^{-2}$ *M* sodium dihydrogen phosphate. The aqueous component was adjusted to pH 2.75 prior to addition of acetonitrile. The chromatographic eluent was pumped at 3.0 ml/min, while the derivatizing reagent solution was pumped at 1.2 ml/min. The fluorometric detector was operated at an excitation wavelength of 229 nm with an emission cutoff filter of 418 nm. The full scale of the ordinate is 1 μA.

Fig. 2. Chromatogram of a derivatized standard solution of trimethyllysine, containing 5.0 nmol/ml trimethyllysine in 2% BSA, which included the internal standard, triethyllysine. The chromatographic conditions were as described in Fig. 1. Peaks: 1 = trimethyllysine; 2 = triethyllysine.

deviation (R.S.D.) of 6%. The R.S.D. in trimethyllysine:triethyllysine peak height ratios obtained for five replicate injections of a single prepared standard solution containing 2 nmol/ml trimethyllysine was 5.7%. The R.S.D. observed in three replicate determinations of a single plasma specimen containing 2.0 nmol trimethyllysine per ml plasma was 4.9%. For three determinations of a kidney specimen containing 2.1 nmol trimethyllysine per g kidney, the R.S.D. was 5.5%.

Fig. 1 is a chromatogram obtained upon preparation of a 1-ml aliquot of the 2% (w/v) aqueous solution of bovine serum albumin employed for preparation of the standards. Fig. 2 is a chromatogram of a standard solution containing 5 nmol/ml trimethyllysine, and triethyllysine. A typical chromatogram obtained upon preparation of a rat plasma specimen is shown in Fig. 3, while a chromatogram obtained after preparation of a rat muscle specimen is reproduced in Fig. 4.

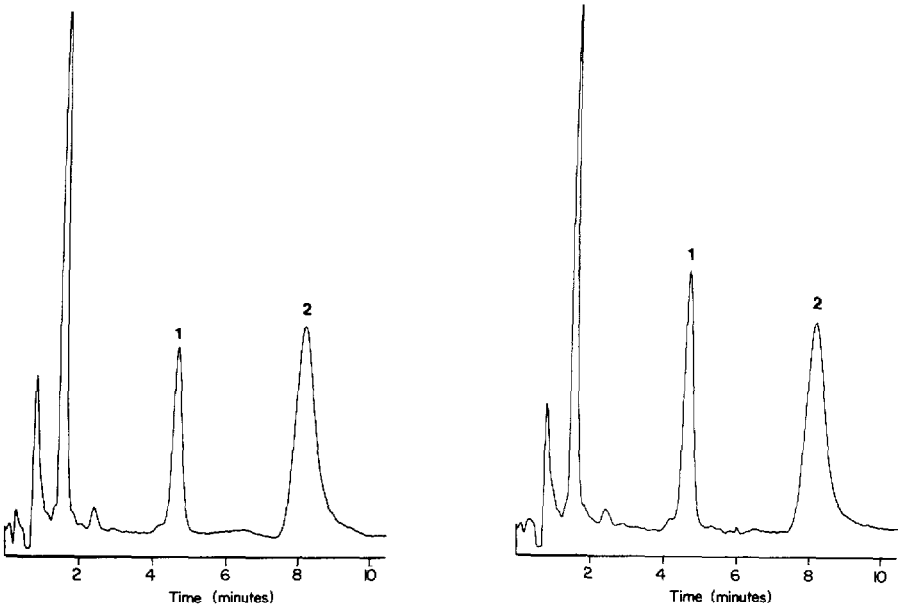

Fig. 3. Chromatogram of a prepared derivatized rat plasma specimen containing 2.0 nmol/ml free trimethyllysine. The chromatographic conditions were as described in Fig. 1. Peaks: 1 = trimethyllysine; 2 = triethyllysine.

Fig. 4. Chromatogram of a prepared derivatized rat muscle specimen calculated to contain 16.3 nmol/g free trimethyllysine. The chromatographic conditions were as described in Fig. 1. Peaks: 1 = trimethyllysine; 2 = triethyllysine.

TABLE II

FREE TRIMETHYLLYSINE IN VARIOUS TISSUES AND PLASMA OF RAT

Five rats were killed by decapitation. Whole blood was collected immediately in heparinized tubes and chilled on ice. Tissues to be removed (liver, kidney, skeletal muscle) were frozen between aluminum blocks which had been cooled in a dry ice—acetone bath. Samples were processed and analyzed as described in the text. Values are expressed as the mean ± the standard error of the mean for four replicate determinations upon skeletal muscle and five replicate determinations upon each of the other tissues.

Tissue	Free trimethyllysine
Plasma (nmol/ml)	1.9 ± 0.1
Liver (nmol/g)	3.2 ± 0.2
Kidney (nmol/g)	2.7 ± 0.1
Muscle (nmol/g)	19.3 ± 1.2

Rats fed a trimethyllysine limiting diet (7.9 nmol trimethyllysine per g diet) for seven days were stunned and decapitated. Blood was collected, and specific organs were removed for determination of free trimethyllysine. The plasma concentration of trimethyllysine in the rat is similar to that found in the chicken [20] and about twice that found in the dog [21]. Concentrations of free trimethyllysine in rat liver, kidney, and skeletal muscle are low, amounting to approximately 1% of free lysines in these tissues [22]. These data are

summarized in Table II. Results of our study of the changes in free trimethyllysine concentrations in the rat during starvation will be reported elsewhere [23].

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