Journal of Chromatography, 380 (1986) 285—299
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3163

IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF 6-N,N,N-TRIMETHYLLYSINE IN PLASMA AND URINE: BIOMEDICAL APPLICATION OF CHROMATOGRAPHIC FIGURES OF MERIT AND AMINE MOBILE PHASE MODIFIERS

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(First received October 18th, 1985; revised manuscript received March 11th, 1986)

SUMMARY

An internally standardized method for the determination of 6-N,N,N-trimethyllysine in human plasma, human urine, rat plasma, rat urine and hydrolyzed rat urine is described. This methylated amino acid and the procedural internal standard 6-N,N,N-triethyllysine were isolated from the sample matrices using short ion-exchange columns and detected following high-performance liquid chromatography using a postcolumn reaction (*o*-phthalic-dicarboxaldehyde—2-mercaptoethanol) and fluorometric detection. The reliable detection limit for 6-N,N,N-trimethyllysine was 0.2 nmol/ml in 200 μl of human plasma. The chromatographic separation exploits the unique properties of a novel tertiary amine mobile phase modifier, 3-(N,N-dimethylamino)-1,2-propanediol. The capacity factor and "Chromatographic Figures of Merit" (including peak asymmetry and relative system efficiency) were calculated for the chromatographic peak representing 6-N,N,N-trimethyllysine in over 2200 injections made while evaluating 900 biological specimens.

INTRODUCTION

The methylated amino acid 6-N,N,N-trimethyllysine (TML) is the biosynthetic precursor of the essential mitochondrial fatty acyltransferase cofactor *l*-carnitine [*l*-4-(N,N,N-trimethylammonio)-3-hydroxybutanoate] [1]. Investigations concerned with the biosynthesis or metabolism of carnitine often require evaluation of the TML content in various biological tissues and fluids.

Lysine and/or its 6-N-methylated derivatives can be determined by one of several methods exploiting ion-exchange chromatography and incorporating

0378-4347/86/$03.50

amino acid analysis [2—11]. Because these methods are non-specific and time-consuming, we developed a high-performance liquid chromatographic (HPLC) method for the determination of TML using precolumn derivatization with 1-fluoro-2,4-dinitrobenzene and successfully performed TML assays in human urine [12] and determinations of peptide-linked TML in tissue specimens [13]. Determination of free TML in rat plasma and tissues required higher sensitivity; therefore we developed a second HPLC method using post-column reaction with *o*-phthalicdicarboxaldehyde—2-mercaptoethanol and fluorometric detection [14]. Although twenty times more sensitive, this post-column derivatization method proved to be difficult to perform on some unusual samples. We, therefore, made major improvements in that procedure, enabling us to fully exploit its potential.

It has been suggested [15, 16] that the addition of amines to eluents used for chromatography on bonded silica reversed-phase chromatographic media masks residual silanol groups which otherwise strongly interact with sample constituents containing basic (particularly amine) functional groups. Encouraged by our success using so-called amine modifiers in chromatographing 4′-bromophenacyl esters of the ω-trimethylammonio carboxylates carnitine, butyrobetaine, and betaine [17], we developed a new chromatographic eluent exploiting the unique properties of an amine modifier for our improved fluorometric TML determination method. The new eluent and refinements in our existing *o*-phthalicdicarboxaldehyde—2-mercaptoethanol postcolumn derivatization system permitted a ten-fold reduction in the amount of plasma sample required for analysis. This allowed more rapid and convenient sample preparation and increased flexibility in experimental design. The stability of this improved HPLC system permitted unattended automated analyses.

To monitor the quality of the chromatographic procedure throughout the duration of the study, several peak descriptors and "Chromatographic Figures of Merit" (CFOM) were routinely calculated for the TML peak in the various analyses performed. The traditional peak descriptors calculated include k' (capacity factor) and peak height. The graphic method of Foley and Dorsey [18] for characterization of exponentially modified Gaussian signals [19, 20] permitted convenient calculation of CFOM for large numbers of experimental samples.

In this paper, we describe the improved HPLC determination of TML which was applied to over 900 human plasma, human urine, rat plasma, rat urine, and hydrolyzed rat urine specimens. We also discuss the use, in a more general context, of CFOM and tertiary amine mobile phase modifiers in HPLC analytical methods.

EXPERIMENTAL

Equipment

The liquid chromatograph consisted of a Model 6000A pump, WISP-710B automatic sampler, and an RCM-100 radial compression module (RCM-100) purchased from Waters Assoc. (Milford, MA, U.S.A.). The chromatographic separation was accomplished using a 10 × 0.5 cm plastic cartridge containing Radial-Pak C_{18} of 10 μm particle diameter (Waters). The chromatographic

column was protected by a 5 × 0.4 cm precolumn packed with Co:Pell ODS (Whatman, Clifton, NJ, U.S.A.). The postcolumn derivatization solution was delivered by an Eldex Labs. (Menlo Park, CA, U.S.A.) Model 1001/E-120-S high-pressure pump and was introduced into the eluent stream through a zero dead volume mixing T-fitting (Waters). A 50 × 0.023 cm coil of steel tubing provided a short reaction period prior to detection. The chromatographic column RCM unit (contained by a watertight plastic sleeve) and the post-column reaction coil were maintained at constant temperature by immersion in a 35°C circulating water bath (MGW Lauda, Model B-1, Curtin Matheson Scientific, Houston, TX, U.S.A.). A fluorescence detector Model SF-970 (Kratos-Schoeffel Instruments, Ramsey, NJ, U.S.A.) operated at an excitation wavelength of 240 nm and with a 418-nm cut-off emission filter was used for eluent fluorescence measurements. The detector output signal was displayed on a Model 8373-00 chart recorder (Cole Parmer Instrument Co., Chicago, IL, U.S.A.). A Hewlett-Packard (Avondale, PA, U.S.A.) Model 3354C laboratory automation system was used for chromatographic peak identification based on relative retention times, peak area integration, peak height measurement, peak shape characterization, and calculations derived from those measurements. Unattended sampling by the HPLC system was made possible by computer-actuated relays connected to (1) the fluorescence detector photomultiplier overload circuitry for programmed resetting of detector overloads, (2) a solenoid valve—pneumatic actuator controlled slide valve system (Rheodyne, purchased from Alltech Assoc., Deerfield, IL, U.S.A.) for unattended HPLC system flushing, and (3) a power outlet strip for unattended HPLC system shut-off. A scintillation spectrometer (PRIAS Model, Packard Instruments, Downers Grove, IL, U.S.A.) was used for liquid scintillation counting.

Materials

Acetonitrile (UV grade, Burdick & Jackson Labs., Muskegon, MI, U.S.A.) was filtered through nylon membranes of 0.45 μm pore diameter. Reagent-grade water was prepared by passage through a Milli-Q reagent-grade water system (Millipore, Bedford, MA, U.S.A.). Sodium dodecyl sulfate was purchased from Gallard Schlesinger (Carle Place, NY, U.S.A.). Sodium phosphate (monobasic), sodium hydroxide, ammonium hydroxide, perchloric acid (60%), and phosphoric acid (85%) were purchased from Fisher Scientific (Cleveland, OH, U.S.A.). 3-(N,N-Dimethylamino)propane was purchased from Ames Labs. (Milford, CT, U.S.A.). 3-(N,N-Dimethylamino)-1-propanol and 3-(N,N-dimethylamino)-1,2-propanediol were purchased from Aldrich (Milwaukee, WI, U.S.A.). All three tertiary amines were distilled under vacuum from potassium hydroxide to remove contaminants (presumably primary amines) which otherwise create intense background fluorescence after postcolumn reaction. Boric acid, *o*-phthalicdicarboxaldehyde, the polyether surfactant Brij-35, and 2-mercaptoethanol were purchased from Aldrich.

TML, [^{14}C-methyl]-6-N,N,N-trimethyllysine ([^{14}C-methyl]TML), and 6-N,N,N-triethyllysine (TEL) were synthesized as described [1, 12, 13]. N^G,N'^G-Dimethylarginine was purchased from Calbiochem (San Diego, U.S.A.). Dowex-1 X8 (200—400 mesh, Cl^-) and Dowex-50 X8 (200—400 mesh, H^+) were purchased from Sigma (St. Louis, MO, U.S.A.) and converted into OH^- and

NH_4^+, respectively, according to instructions published by Bio-Rad Labs. (Richmond, CA, U.S.A.). Conversion of the anion-exchange resin must be continued until tests for Cl^- in the column effluent are negative.

Preparation of reference solutions

TML chloride salt (0.0056 g, $2.5 \cdot 10^{-5}$ mol) was added to 50 ml reagent-grade water to obtain a 500 nmol/ml stock solution. All TML working solutions were prepared by dilutions of this stock solution. TEL (isolated as a syrup after synthesis) was diluted 1:6400 (w/v). This stock solution was standardized [21] relative to TML standard solutions and found to contain 410 ± 20 nmol TEL/ml (n=10). All TEL reference solutions were made by dilutions of this standardized stock solution. The precision of this determination was sufficient, since the TEL solution concentration need not be determined exactly for its use as a procedural internal standard. These TML and TEL standard solutions were found to be greater than 97% chromatographically pure under the HPLC chromatographic conditions (*Chromatography for TML determination*).

Animals

Male Sprague—Dawley rats (260-280 g) purchased from Zivic-Miller (Allison Park, PA, U.S.A.) were fed a purified diet [22] which is low in carnitine and TML [23]. The rats were housed in wire-bottomed metabolic cages and were fed the diet for one week. Urine was collected from one group of rats fed for an additional three days. Another group was fasted for up to five days with daily urine collection. At designated times, rats were killed by stunning, decapitated, and blood was collected in heparinized beakers.

Human subjects

Plasma and urine samples were available from current and previous studies [24, 25].

Sample preparation for TML determination

Plasma (human and rat). In a 1.5-ml (13 × 33 mm) polypropylene conical tube were combined 200 µl heparinized plasma or TML standard solution (0.2—2 nmol/ml for human, 0.5—5 nmol/ml for rat) and 120 µl TEL internal standard solution (10 nmol/ml). The tube was vortexed and 150 µl of 10% perchloric acid was added. The tube was vortexed again and then centrifuged at 10 000 *g* for 2.5 min. The resulting supernatant was applied to the cation/anion resin sample preparation system described below.

Human urine. In a 1.5-ml (13 × 33 mm) polypropylene conical tube were combined 100 µl urine or TML standard solution (10—100 nmol/ml) and 45 µl TEL internal standard solution (410 nmol/ml). The tube was vortexed and 125 µl of its contents was applied to the cation/anion resin sample preparation system described below.

Rat urine. In a 1.5-ml (13 × 33 mm) polypropylene conical tube were combined 200 µl urine or TML standard solution (5—50 nmol/ml for fed rats, 0.5—10 nmol/ml for fasted rats) and 120 µl TEL internal standard solution (140 nmol/ml for fed rats, 20 nmol/ml for fasted rats). The tubes were vortexed, and 300 µl of the contents was applied to the cation/anion resin sample preparation system described below.

Acid-hydrolyzed rat urine. In a 12 × 100 mm glass screw-top test tube were combined 100 μl urine or TML standard solution (15—150 nmol/ml), 60 μl TEL internal standard solution (410 nmol/ml), and 400 μl concentrated hydrochloric acid. The tube was sealed with a PTFE-lined screw cap, vortexed, and placed on a heating block at 105°C for 18 h. The solution was evaporated to dryness, reconstituted in 200 μl of 0.1 *M* hydrochloric acid, and applied to the cation/anion resin sample preparation system described below.

Cation/anion resin sample preparation system

Two ion-exchange chromatographic operations were required to remove interfering compounds from biological samples. Each sample was applied to a 3.5 × 0.5 cm column of Dowex-50 X8 (200—400 mesh, NH_4^+, water-washed) cation-exchange resin contained by a pasteur pipet. The column was washed with 2 ml of reagent-grade water, then placed directly above a 3.5 × 0.5 cm column of Dowex-1 X8 (200—400 mesh, OH^- water-washed) anion-exchange resin also contained by a pasteur pipet. TML and TEL, internal standard, were eluted from these two columns with 4 ml of 2.2 *M* ammonium hydroxide. The first 0.5 ml was discarded; the last 3.5 ml was collected in a 12 × 75 mm polypropylene test tube. Each sample tube was transferred to a 40°C water bath and the contents evaporated to dryness by a stream of compressed air. The samples were reconstituted in 100 μl of 0.085% (v/v) phosphoric acid before injection into the HPLC system.

Chromatography for TML determination

The chromatographic eluent was prepared by dissolving 0.72 g ($2.5 \cdot 10^{-3}$ mol) sodium dodecyl sulfate, 4.14 g ($3 \cdot 10^{-2}$ mol) sodium phosphate (monobasic), and 2.38 g ($2 \cdot 10^{-2}$ mol) 3-(N,N-dimethylamino)-1,2-propanediol in 820 ml reagent-grade water and adjusting the solution pH to 3.5—3.2 (depending on the history of use of the analytical column) with concentrated phosphoric acid. This solution was filtered through a 0.45-μm pore diameter membrane and added to 180 ml filtered acetonitrile with thorough mixing. The derivatization reagent solution was prepared by combining 500 ml of 0.5 *M* boric acid (pH adjusted to 10.4 with sodium hydroxide pellets, then filtered), 0.3 g *o*-phthalicdicarboxaldehyde, 10 ml Brij-35 solution (3%, w/v, in 95% ethanol), and 0.5 ml 2-mercaptoethanol.

The chromatographic eluent was placed in an aluminum foil-covered solvent reservoir, continually sparged with nitrogen, and pumped through the HPLC system at a flow-rate of 4.0 ml/min. The postcolumn derivatization reagent solution was placed in a foil covered solvent reservoir, continually sparged with nitrogen, and pumped through foil-covered solvent lines into the eluent stream at a flow-rate of 2 ml/min. Aliquots (5—80 μl) of isolated specimens were injected into the chromatograph.

Quantification

Standard curves of TML/TEL peak height ratios versus concentration were established daily. The TML concentration in experimental samples was interpolated from a least-squares regression line through standard data points. All standards and experimental samples were analyzed in duplicate.

Recovery and reproducibility studies

Recovery of TML from the cation/anion resin sample preparation system was demonstrated by applying an aliquot of a TML standard solution to each of six columns and eluting as described above. An aliquot of TEL standard solution was added to each of these six samples after ion-exchange chromatography to serve as an external standard for the HPLC experiment. These samples were injected into the HPLC system, and the TML/TEL peak height ratios were determined. These ratios were compared to peak height ratios of six replicate recovery standards generated using identical aliquots of the same standard solutions. The mean of the six experimental sample peak height ratios divided by the mean of the six recovery standard peak height ratios multiplied by 100% expresses the recovery. The recovery of TEL was also determined using TML as the external standard.

The distribution of TML between a protein precipitate and its perchloric acid supernatant was determined by adding 120 μl of an aqueous solution containing [^{14}C-methyl]TML (100 000 dpm) to each of four 200-μl rat plasma samples, precipitating the protein as described under *Plasma (human and rat)*, and determining the radioactivity contained in the supernatant. This was compared to the radioactivity found in four replicate 120-μl aliquots (recovery standards) of the [^{14}C-methyl]TML solution. The percentage of TML recovered in the plasma supernatant can be expressed as the ratio of the mean of the four experimental sample dpm values divided by the mean of the four recovery standard dpm values multiplied by 100%. The difference in recovered radioactivity between samples and recovery standards could be recovered by resuspending the sample protein precipitate pellet in formic acid and determining the contained radioactivity. The sum of supernatant plus protein pellet radioactivity was equal to recovery standard radioactivity.

Two representative samples of human plasma, human urine, rat urine, and hydrolyzed rat urine were analyzed in replicate groups of six on three separate days to determine the sample-to-sample and day-to-day reproducibility. A representative hydrolyzed rat urine sample carried through the analytical procedure was injected into the HPLC system ten times to establish injection-to-injection reproducibility.

Chromatographic peak characterization

The HP-3354C data system is a Series HP-1000 minicomputer running under a custom operating system designed for low-frequency real-time chromatographic data acquisition. A BASIC program was developed for assessment of CFOM of exponentially modified Gaussian (EMG) peaks according to an algorithm and equations described by Foley and Dorsey [18]. The functional elements of the program were tested with standard data [26] designed to simulate the fundamental parameters t_G (retention time of the underlying parent Gaussian function), σ_G (standard deviation of the parent Gaussian function), and τ (time constant of the exponential modifier) of the peaks of interest in this study. These parameters were extracted from the simulated data with relative errors of $\pm 1\%$, $\pm 2\%$, and $\pm 7\%$ for t_G, σ_G, and τ, respectively. The noted inaccuracy seemed to originate with the imprecision (± 0.5 s total at 2 Hz sampling frequency) associated with peak width measurement and from the round-

off error inherent in single precision arithmetic. The program incorporated a sensitive test for distortion of the leading and trailing edges of chromatographic peaks. In this application, such distortions indicate the presence of incompletely resolved minor sample constituents. Peaks which failed this test were rejected from further CFOM assessment.

Amine mobile phase modifier study

The chromatographic mobile phases were prepared by dissolving 2.88 g (10^{-2} mol) sodium dodecyl sulfate, 1.38 g (10^{-2} mol) sodium phosphate (monobasic), and 10^{-2} mol of the appropriate tertiary amine in 900 ml reagent-grade water and adjusting the solution pH to 5 with concentrated phosphoric acid. To simulate an eluent without added tertiary amine, an eluent was prepared in which sodium hydroxide (10^{-2} *M*) was substituted for the tertiary amine mobile phase component to maintain constant ionic strength. These solutions were filtered through 0.45-μm pore diameter membranes and each was added to 100 ml filtered acetonitrile with thorough mixing. The eluents and derivatization reagent solution were employed as described above (*Chromatography for TML determination*). The chromatographic system was washed with 100 ml eluent prior to duplicate injections of each individual N-methylated amino acid. Peak descriptors were determined and the system was washed with 100 ml (20%, v/v) acetonitrile between each eluent change.

RESULTS AND DISCUSSION

The determination of TML in biological samples is generally performed in conjunction with quantification of a mixture of amino acids using an amino acid analyzer. Typically [11], 50 μg of hydrolyzed protein are required and the analysis time is approximately 3.5 h per sample. Since our interest in amino acids was limited to TML, our methods development strategy has been to develop rapid and specific TML determination methods rather than generating lengthy and time-consuming amino acid profiles. We have previously shown rapid determination of TML to be feasible [12—14], but as our research goals became more demanding, we found it necessary to improve our determination method.

Our earlier method for TML determinations [14] was poorly suited to large-scale application in several respects. First, the chromatographic separation proved unsuitable for some unusual but clinically important human urine specimens. Second, the daily HPLC sample throughput was limited to ten samples plus a five-point standard curve (all in duplicate) due to manual injection and the required constant operator attention to the fluorescence detector to readjust the drifting baseline and to reset its photomultiplier overload circuitry. This limited weekly throughput to 30 experimental samples. Third, the initial ion-exchange sample preparation required resin columns with a total volume of 5 ml, generating an ammonium hydroxide effluent volume of 8 ml (which then had to be evaporated). Fourth, determination of TML in plasma required a sample volume of 4 ml (2 ml in duplicate). Last, the system required long equilibration time, and even when equilibrated exhibited noticeable baseline drift.

TABLE I

METHOD VALIDATION STUDIES ON THE HPLC ANALYSIS OF 6-N,N,N-TRIMETHYLLYSINE

Sample	Standard curve range (nmol/ml)	Standard curve slope*	Standard curve *y*-intercept*	Standard curve linear regression coefficient*	Sample-to-sample reproducibility** (*n*=6) (nmol/ml)	Day to-day reproducibility** (*n*=6, each of three days) (nmol/ml)
Human urine	10 —100	0.0176 ± 0.0004 (*n* = 3)	0.021 ± 0.002 (*n* = 3)	0.9991 ± 0.0002 (*n* = 3)	71.6 ± 0.9 30.8 ± 0.4	70 ± 6 33 ± 3
Human plasma	0.2—2.0	0.88 ± 0.06 (*n* = 3)	0.2 ± 0.1 (*n* = 3)	0.988 ± 0.002 (*n* = 3)	0.8 ± 0.1 0.5 ± 0.0	0.7 ± 0.1 0.5 ± 0.1
Rat urine***	5 —50 0.5—10.0	0.0371 ± 0.0009 0.23 ± 0.02 (*n* = 4)	−0.03 ± 0.02 0.04 ± 0.02 (*n* = 4)	0.997 ± 0.001 0.998 ± 0.002 (*n* = 4)	13.3 ± 0.4 0.4 ± 0.1	14.0 ± 1.0 0.5 ± 0.1
Rat urine (hydrolyzed)	15—150	0.0104 ± 0.0005 (*n* = 6)	0.01 ± 0.02 (*n* = 6)	0.993 ± 0.004 (*n* = 6)	100 ± 4 22.0 ± 1.0	101 ± 3 22.2 ± 0.9

*These were calculated using standard solutions of 6-N,N,N-trimethyllysine and the procedural internal standard 6-N,N,N-triethyllysine from the ratios of their chromatographic peak heights.

**These values were interpolated using the least-squares regression line through standard data points calculated from the ratios of their chromatographic peak heights. For each sample matrix, two samples were chosen for the reproducibility studies representative of a high (the top sample) and low (the bottom sample) sample concentration.

***Two standard curves were necessary to cover the sample concentration range in rat urine.

We noticed that the laboratory underwent room temperature changes as a function of season and time of day. Since the postcolumn reaction rate is temperature-dependent to some degree, fluctuation in room temperature may have caused changes in background fluorescence. The chromatographic column compression unit and postcolumn reactor were therefore placed in a waterbath thermostat. The substituted fluorescent isoindole derivative [27] is readily oxidizable and amenable to oxidative electrochemical detection [28]. We therefore sought to minimize changes in the oxygen content of both eluent and derivatizing agent with nitrogen sparging. We also noticed that overnight chromatographic runs carried out in darkness with nitrogen sparging and water bath temperature control exhibited less baseline drift than similar chromatographic runs conducted during the daytime. The eluent reservoirs and solvent delivery lines therefore were covered with aluminum foil to exclude light.

These measures resolved the shortcomings of our previous method. The new chromatographic eluent permitted analysis of all of our urine and plasma specimens. The use of an HPLC automatic sampler and the installation of computer-actuated relays allowed unattended overnight sampling through programmed resetting of the fluorescence detector overload circuitry, HPLC system wash-out, and HPLC system shut-down. Placing the HPLC column and postcolumn reaction coil in a water bath, covering reservoirs and solvent lines with foil, using a longer fluorescence excitation wavelength, using UV-grade acetonitrile, and nitrogen sparging of the HPLC eluent and postcolumn reaction solutions had the combined effect of improving the HPLC detection sensitivity and eliminating baseline drift problems. These improvements in the HPLC system allowed a ten-fold reduction in the amount of plasma required for analysis and thus a two-fold reduction in the amount of ion-exchange resin sample preparation column effluent generated. As a result, our rate of TML determination was quadrupled. During this study, one operator was able to complete 120 determinations with two five-point standard curves per week, all in duplicate.

The stability of TML in human plasma was examined over an eight-month period. Approximately 50 ml of blood were drawn from a normal human subject. The plasma was isolated and stored in a freezer at −20°C. During the following eight months, the plasma TML content of this specimen was determined 38 times, yielding a value of 0.5 ± 0.1 nmol/ml. The stability of TML in human urine was investigated by comparing TML concentration values obtained using our earlier spectrophotometric HPLC determination method [12] and those obtained four years later with the current method. Six samples with TML concentrations ranging between 9 and 41 nmol/ml were compared and found to yield identical values, ± 2 to 4 nmol/ml. These results are consistent with our reproducibility studies (see Table I).

During our experience with 2200 analyses, we saw a gradual change in the HPLC column performance. The elution order among different peaks in the chromatogram remained constant, but absolute retention and resolution tended to decrease. We began this work with an eluent pH of 3.5 but found it necessary, convenient, and satisfactory to lower the pH first to 3.3 and then to 3.2 to maintain and then increase chromatographic resolution for determination of TML in human plasma. We found that reproducible chromatographic retention

of TML is highly dependent on reproducible pH measurement in this range. We found it necessary to use the same glassware and pH meter, and to compensate for day-to-day solution temperature variation during pH adjustment to ensure reproducible eluent preparation.

Studies were undertaken to determine the precision of this analytical procedure. Recovery of TML and TEL from the cation/anion resin sample preparation system was 99 ± 5% and 93 ± 5%, respectively, (mean ± S.D., $n = 6$). The distribution of TML between the perchloric acid supernatant and its protein pellet was 88.7 ± 4.6% and 9.8 ± 1.1%, respectively (mean ± S.D., $n = 4$). Replicate injections of a representative hydrolyzed rat urine specimen resulted in a TML value of 22.0 ± 0.4 nmol/ml (mean ± S.D., $n = 10$). Table I summarizes data from sample-to-sample and day-to-day reproducibility studies. These method validation studies were undertaken to define the degree of precision which can be expected from this procedure. They were performed on representative samples and embody worst case rather than best case assumptions. We are confident that these results could be reproduced elsewhere.

During this study, we needed a basis for evaluation of both the quality of the chromatographic separation of the compounds of interest and the performance of analytical columns throughout their useful lifetimes. Several methods of peak and column performance characterization have been reviewed recently [29]. These authors confirmed earlier findings [30] that peak characterization calculation procedures derived from assumptions of purely Gaussian peak shape may significantly overestimate the separation efficiency of the tailing peaks often encountered during chromatographic experiments. The EMG model proposed by Gladney et al. [31] and subsequently examined by others [32, 33] is now well accepted. This model holds that a chromatographic peak is well represented as the convolution of a Gaussian probability distribution with an exponential decay process.

Several peak characterization procedures were tested. When applied to standard data sets, statistical moment calculations gave nearly exact results regardless of actual peak shape, but this calculation procedure is known to be overly sensitive to uncertainty in assignment of peak start and stop times [34], and to noise present in experimental chromatograms. A hybrid statistical moment summation and iterative search procedure developed by Yau [35] was examined. This also worked well with standard data sets, but failed often when applied to experimental data collected at low frequency.

Recently, Foley and Dorsey [18] described and validated a new method for calculation of a series of CFOM. They adduced a series of least-squares fitted equations derived from the EMG peak model. Their approach is easily understood and easily programmed. Only the peak retention time (t_R) and the times on both the leading and trailing edges of peaks at which the signal amplitude reaches 10% of that at t_R must be determined. We tested this calculation method and found it to behave well when applied to actual chromatographic data. Accuracy improves with increasing peak t_R, as the ±0.1 s imprecision in assignment of peak t_R and the ±0.5 s total imprecision in identification of time at 10% of peak height become small in relation to the peak variance and the exponential modifier time constant. The CFOM calculation procedure is comparatively insensitive to low-level noise in the detector output signal.

The peak descriptors and CFOM of EMG peaks most useful to chromatographers include k' (the capacity factor), σ_G (the standard deviation of the parent Gaussian function), τ (time constant of the exponential modifier), the ratio τ/σ_G (a fundamental index of peak asymmetry), N_{sys} (efficiency of the separation, including chromatographic and non-chromatographic contributions to band broadening), N_{max} (theoretical maximum separation efficiency in the absence of band broadening contributors), and RSE (relative system efficiency, N_{sys}/N_{max}). Simple calculation of τ/σ_G values is especially useful during separation development. This CFOM provides an objective criterion for assessment of peak shape changes following adjustment of the mobile phase composition.

Peak descriptors and CFOM data obtained in this study are presented in Table II. They are typical of results obtained during chromatographic experiments with the named sample types. The calculation algorithm we employed incorporated a sensitive test for distortions of the leading and trailing edges of chromatographic peaks which perturb CFOM calculations. Peaks were rejected by the program when incompletely resolved from other endogenous sample constituents found in some specimens. The tabulation of capacity factors and retention times (with relative standard deviations of 1.4%) demonstrates the excellent stability of the chromatographic system during experimental trials of as many as 18 h duration. For chromatography of human plasma, the pH of the chromatographic eluent was decreased from 3.5 to 3.3. This provided better resolution of TML and an often larger endogenous sample constituent peak with a concommitant increase in absolute retention to 3.8 min. Small changes in retention as a function of small trial-to-trial variation in eluent preparation were noted. This was minimized by strict attention to eluent pH adjustment with compensation for day-to-day changes in solution temperature at the time of eluent preparation. τ/σ_G is a useful index of peak asymmetry. We have found that values of 1—2 are typical in isocratic reversed-phase chromatograms. Greater peak asymmetry is often observed in reversed-phase ion-pair separations of basic and quaternary ammonio compounds [36].

TABLE II

SELECTED PEAK CHROMATOGRAPHIC DESCRIPTORS FOR 6-N,N,N-TRIMETHYLLYSINE IN BIOLOGICAL SAMPLES

Sample matrix	Number of injections	Number of injections suitable for analysis	Capacity factor k' (mean ± S.D.)	Retention time t_R (mean ± S.D.) (min)	τ/σ_G peak asymmetry descriptor (mean ± S.D.)	Relative system efficiency (N_{sys}/N_{max}) (mean ± S.D.)
Human urine	66	64	5.85 ± 0.07	2.74 ± 0.03	0.7 ± 0.6	0.7 ± 0.2
Human plasma*	110	97	8.50 ± 0.05	3.80 ± 0.02	0.9 ± 0.6	0.6 ± 0.2
Hydrolyzed rat urine	74	74	6.66 ± 0.05	3.06 ± 0.02	0.7 ± 0.1	0.7 ± 0.1
Rat urine (fed)	55	48	7.3 ± 0.1	3.33 ± 0.04	0.7 ± 0.2	0.7 ± 0.1
Rat urine (fasted)	98	98	6.88 ± 0.07	3.15 ± 0.03	1.3 ± 0.7	0.5 ± 0.2
Rat plasma	88	83	6.12 ± 0.05	2.85 ± 0.02	0.6 ± 0.4	0.8 ± 0.1

*The chromatographic eluent pH was lowered in human plasma to 3.3 to improve the resolution.

The low τ/σ_G values (and high RSE values) obtained in this study reflect low contribution to band broadening by both chromatographic and non-chromatographic processes. We noted that during the lifetime of an analytical column, the peak shape and separation efficiency of the TML and TEL peaks changed very little. However, absolute retention of all sample constituents decreased slowly with column use, and column back-pressure at the analytical flow-rate slowly increased. These findings forced replacement of analytical columns after approximately 1500 injections.

The initial objective for modification of our previous TML determination method [14] was to improve the selectivity among various unusual sample constituents which were present in the urine of a patient with an inborn metabolic error. Changes in the content of ion-pairing reagent, organic mobile phase modifier, and pH of the chromatographic eluent did not provide the resolution necessary for TML quantification. The work of Gill et al. [16] demonstrated the use of amine mobile phase modifiers in separation of amine solutes. These workers showed that different amine mobile phase modifiers produce dramatically different effects on the retention of amine solutes. We have employed several hydroxylated amine mobile phase modifiers during our work with carnitine and similar compounds [17]. We systematically studied the effects of the addition of various tertiary amines on the reversed-phase ion-pair elution of selected N-methylated amino acids from the non-endcapped ODS bonded silica chromatographic medium Radial-Pak C_{18} [37]. We found that the presence of hydroxyl functional groups on a tertiary amine mobile phase modifier produces an increase in the retention of amine solutes relative to an eluent containing an unsubstituted tertiary alkylamine yet a decrease in retention when compared to an eluent containing no amine mobile phase modifier at all. Increasing the number of hydroxyls on the tertiary amine mobile phase modifier employed, while controlling all other mobile phase composition variables, increases the retention of amine solutes when compared to the analogous less hydroxyl-substituted tertiary amine mobile phase modifier. Table III summarizes the results of a study in which TML, TEL, and N^G,N'^G-dimethylarginine (DMA) were chromatographed with eluents containing amine mobile phase modifiers. It should be noted that the effect of an amine mobile phase modifier is different for different amine solutes, as demonstrated by the reversal in the elution order of TEL and DMA when using no amine mobile phase modifier (sodium hydroxide is used to control ionic strength) versus the unsubstituted tertiary amine. Sequential substitution of the tertiary amine mobile phase modifier with first one and then a second hydroxyl functional group resulted in elution patterns between these two extremes. Unlike the previous study [16], we did not perceive an increase in peak symmetry when using amine mobile phase modifiers. The τ/σ_G and RSE values calculated (Table III) were similar for all the eluents. We concluded that amine mobile phase modifiers can provide a fourth variable, in addition to pH, ion-pairing reagent, and organic mobile phase modifier, for manipulation while performing complex separations of quaternary amines. The results of studies like that summarized in Table III led to the formulation of the mobile phase employed in our current TML determination method.

Fig. 1 is a chromatogram of a human urine specimen carried through the

TABLE III

SELECTED PEAK DESCRIPTORS DETERMINED FOR 6-N,N,N-TRIMETHYLLYSINE, 6-N,N,N-TRIETHYLLYSINE, AND N^G,N'^G-DIMETHYLARGININE WHEN CHROMATOGRAPHED IN THE PRESENCE OF NON-, MONO-, AND DI-HYDROXY-SUBSTITUTED 3-DIMETHYLAMINOPROPANE AMINE MOBILE PHASE MODIFIERS

Chromatographic conditions: The mobile phases contained 10^{-2} *M* each of sodium dodecyl sulfate, sodium phosphate (monobasic), and amine modifier in acetonitrile—water (10:90, v/v). The aqueous component was adjusted to pH 5.0 prior to the addition of acetonitrile. The chromatographic eluent was pumped at 4.0 ml/min.
t_R = Retention time (in min); k' = capacity factor; τ/σ_G = Chromatographic Figures of Merit peak asymmetry factor; RSE = Chromatographic Figures of Merit relative system efficiency (N_{sys}/N_{max}).

Amine modifier	6-N,N,N-Trimethyllysine				6-N,N,N-Triethyllysine				N^G,N'^G-Dimethylarginine			
	t_R	k'	τ/σ_G	RSE	t_R	k'	τ/σ_G	RSE	t_R	k'	τ/σ_G	RSE
3-N,N-Dimethylaminopropane	3.1	6.7	0.8	0.6	4.3	9.8	0.5	0.8	5.1	11.8	0.4	0.9
3-N,N-Dimethylamino-1-propanol	4.8	10.9	0.7	0.6	9.8	23.6	0.6	0.7	10.0	23.9	0.5	0.8
3-N,N-Dimethylamino-1,2-propanediol	5.8	13.5	0.6	0.7	14.3	34.8	0.7	0.6	13.0	31.4	0.3	1.0
Sodium hydroxide*	8.8	21.0	0.9	0.5	27.0	66.5	1.0	0.5	18.8	46.0	0.3	1.0

*Sodium hydroxide (10^{-2} *M*) was used in place of a tertiary amine to simulate the chromatography resulting from the lack of an amine modifier.

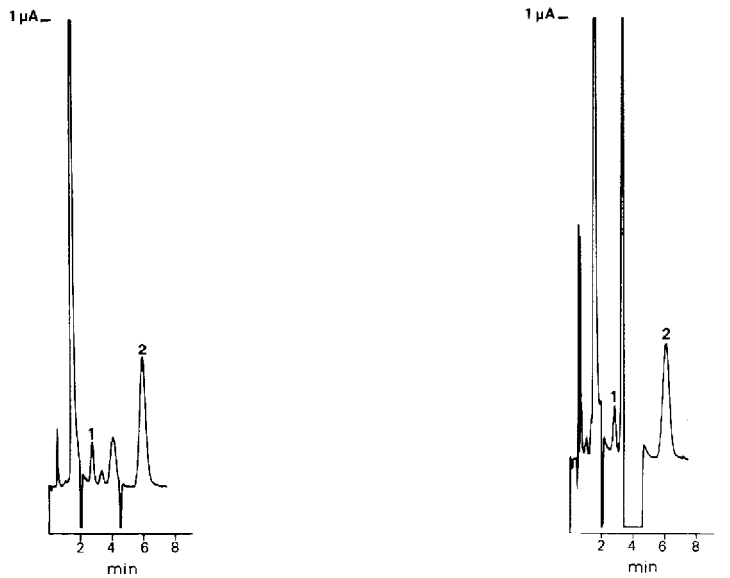

Fig. 1. Representative chromatogram of TML in human urine using the described procedure. The column used was a 10 × 0.5 cm I.D. radially compressed cartridge of Radial-Pak C_{18} (10 μm particle diameter). The chromatographic eluent was $2.5 \cdot 10^{-2}$ *M* sodium dodecyl sulfate—$3 \cdot 10^{-2}$ *M* sodium phosphate (monobasic)– $2 \cdot 10^{-2}$ *M* 3-(N,N-dimethylamino)-1,2-propanediol in acetonitrile– water (18:82, v/v). The aqueous component was adjusted to pH 3.5 prior to the addition of acetonitrile. The chromatographic eluent was pumped at 4.0 ml/min, and the postcolumn derivatization solution was pumped at 2 ml/min. The fluorometric detector was operated at an excitation wavelength of 240 nm and with an emission cut-off filter of 418 nm. The injection volume was 5 μl. Peaks: 1 = TML; 2 = TEL, internal standard. TML concentration: 17.4 nmol/ml.

Fig. 2. Representative chromatogram of TML in rat plasma using the described procedure. The chromatographic conditions and peak identification are as described under Fig. 1. The injection volume was 80 μl; TML concentration: 0.7 nmol/ml.

analytical procedure. The injection volume was 5 μl and the TML concentration was determined to be 17.4 nmol/ml. The τ/σ_G value for the TML peak was 0.53 and the RSE was 0.81. This chromatogram clearly shows the highly symmetric shape of the chromatographic peaks. Sensitivity was sufficient.

With only 5% of the isolated sample injected, the TEL internal standard peak amplitude is nearly 30% of full scale. This chromatogram represents 80 pmol TML and 800 pmol TEL injected into the chromatograph.

Fig. 2 is a chromatogram of a rat plasma specimen carried through the analytical procedure. The injection volume was 80 μl and the TML concentration was determined to be 0.7 nmol/ml. The τ/σ_G value for the TML peak was 0.73 and the RSE was 0.66. Although visibly different from chromatograms generated using our earlier method [14], the advantages of speed, enhanced sensitivity, reliability, and ease of sample preparation outweigh any disadvantage of this more complex chromatogram. The large peak between TML and TEL (which triggers the fluorescence detector's overload circuitry) is similar in retention time to that of N^G-methylated arginines. N^G-Methylated arginines are not eliminated from the sample by the cation/anion exchange resin sample preparation system. This chromatogram represents 100 pmol TML and 860 pmol TEL injected into the chromatograph.

ACKNOWLEDGEMENTS

We wish to thank Ms. Deborah Collins for assistance in preparing the manuscript. This work was supported by funds provided by the Veterans Administration Medical Research Program and the National Institutes of Health (AM 15804).

NOTE ADDED IN PROOF

Recently, a paper was published by Kohse et al. [38] using precolumn derivatization for the measurement of TML and requiring 10 ml human plasma per analysis. In contrast, the method reported in our paper requires only 0.2 ml of plasma per analysis. The earlier method of Davis et al. [14] required 2.0 ml of plasma per analysis, not the 100 ml blood incorrectly calculated by Kohse et al. [38].

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