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Note

High-performance liquid chromatographic determination of plasma free trimethyllysine in humans

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N-6-Trimethyllysine (TML), a rare amino acid, has been shown to be a constituent of several mammalian proteins, such as myosin [1], calmodulin [2], and histones [3]. It is formed by post-translational methylation of lysine residues with S-adenosylmethionine [4]. Once released by proteolytic action, it may serve as a precursor for the endogenous biosynthesis of carnitine [5], the essential carrier substance for the transport of long-chain fatty acids across the inner mitochondrial membrane (see ref. 6 for a review).

It has been proposed that the biosynthesis of carnitine may be disturbed in certain pathological conditions [7, 8]. Thus, an evaluation of plasma TML concentrations in various diseased states might be of interest to biochemists and clinicians.

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The presence of free TML in human plasma was demonstrated as early as 1970 [9], but quantification of this amino acid (or other methylated basic amino acids) has been hampered by rather insensitive and lengthy methods involving ion-exchange chromatography or conventional amino acid analysis [10—13]. The considerable volume of blood required was an additional drawback. Recently, Hoppel et al. [14] and Davis and Hoppel [15] described the determination of TML in urine and tissue hydrolysates using a high-performance liquid chromatographic (HPLC) method with fluorometric detection following derivatization with 1-fluoro-2,4-dinitrobenzene; however, the method was too insensitive for measurement of TML concentrations in plasma. In a subsequent report from the same authors [16], a suitable HPLC method with post-column derivatization was proposed for the assessment of free TML in rat plasma. However, the plasma free TML concentration in rats is about tenfold higher than that in humans. In the search for a satisfactory method enabling determination of TML in human plasma, we successfully adapted a recently described method designed for determination of free amino acids [17, 18]. We employed pre-column derivatization with *o*-phthaldialdehyde—3-mercaptopropionic acid (OPA—3-MPA). In the present communication we report its applicability to the measurement of TML, and normal values for plasma TML obtained by using this method in healthy males and females.

EXPERIMENTAL

Chemicals

Trimethyllysine dioxalate was obtained from Calbiochem (San Diego, CA, U.S.A.). Triethyllysine (TEL) was synthesized from 1-N-acetyllysine and iodoethane according to Cox and Hoppel [19]. 1-N-Acetyllysine was from Sigma (St. Louis, MO, U.S.A.). OPA and potassium borate buffer (1.0 *M*, pH 10.4) were purchased from Pierce (Rockford, IL, U.S.A.), and 3-MPA was supplied by Fluka (Buchs, Switzerland). All other chemicals used were of reagent-grade purity and were obtained from E. Merck (Darmstadt, F.R.G.). Ultra-pure water generated with an Elgastat-spectrum water purification system including reverse osmosis, activated carbon and nuclear-grade ionization cartridges (Elga, Lane End, U.K.) was always used in the preparation of reagents or buffers.

Sample preparation procedure

To 10 ml of human heparin plasma, 0.5 μM triethyllysine and 2 ml of 300 g/l perchloric acid were added. After 1 h on ice and following centrifugation at 4000 *g* for 10 min, the pellet was washed twice by resuspension in 2 ml of ice-cold 50 g/l perchloric acid and centrifugation as above. The supernatant and the washings were combined and neutralized by addition of 470 g/l potassium hydroxide. After 30 min on ice, the potassium perchlorate precipitate was removed by centrifugation as above. The supernatant was applied to an ion-exclusion/ion-exchange column (15 ml of AG50 WX8, 200—400 mesh, ammonium form, on top of 15 ml of AG1 X8, 200—400 mesh, hydroxy form). The column was washed with 30 ml of water and 20 ml of 1 *M* ammonia. Trimethyllysine and the internal standard, triethyllysine, were eluted with

50 ml of 1 *M* ammonia. The eluate was evaporated to dryness in a rotary evaporator (temperature of the water-bath 37°C) and the residue was reconstituted in 1 ml of HPLC-quality water.

A 0.23-ml volume of this sample was mixed with 0.23 ml of potassium borate buffer (1.0 *M*, pH 10.4) and filled into a sample vial, which was then placed in the cooled autoinjector (see below).

High-performance liquid chromatography

Apparatus. The HPLC system (LKB, Bromma, Sweden) consisted of two Model 2150 pumps, a Model 2152 controller for gradient programming and a modified Model 2153 autoinjector with a 20-μl filling loop for automated on-line derivatization. Fluorescence was routinely monitored with a Model RF-530 spectromonitor (Shimadzu, Kyoto, Japan) at an excitation wavelength of 330 nm and an emission wavelength of 450 nm with a 12-μl flow-cell and a xenon lamp. Continuous on-line quantitation of the HPLC results was obtained with a Model Chromatopac C-R3 A data processor (Shimadzu).

Column. The HPLC column used was Hyperchrome Shandon Hypersil (3 μm), 250 × 4.6 mm I.D., with a guard-column 10 × 4.6 mm I.D. (Bischoff-Analysentechnik, Leonberg, F.R.G.). The separations were performed at room temperature with a flow-rate of 1.5 ml/min and a pressure of 230 bar.

Solvent gradient. A two-component gradient system was used (solvent A: 5 ml/l tetrahydrofuran in 12.5 m*M* sodium phosphate, pH 7.2; solvent B: 0.5 l/l acetonitrile in 12.5 m*M* sodium phosphate, pH 7.2). Solvents were helium-conditioned and degassed using a Model 2156 solvent conditioner. Gradient fix-points: 0 min: 18% B; 4 min: 18% B; 10 min: 35% B; 11 min: 100% B; 14 min: 100% B; 15 min: 18% B.

Preparation of the derivatization reagent

OPA (25 mg) was dissolved in a mixture of 2 ml of methanol, 5 ml of 1 *M* potassium borate (pH 10.4), and 18 ml of water, and 0.2 ml of 3-MPA was added. The solution was vigorously shaken and stored for at least two days at 4°C in the dark prior to use. Fresh mixtures were prepared each week.

Preparation of the standard solution

A 10-μl volume of a 2.5 m*M* solution of TML, TEL, and arginine (Arg) was diluted with 10 ml of water and 2 ml of potassium borate (1.0 *M*, pH 10.4). Each standard vial was filled with 1 ml of this solution and placed in the cooled autoinjector.

Automated sample injector

An LKB 2153 autoinjector was modified to enable automated pre-column derivatization and subsequent injection. Thus, the normal digitally preset functions of the autoinjector for flush cycles and the injection/fill cycles were changed in digitally preset functions for pump time (90 s) and derivatization time (150 s), respectively.

The on-line derivatization was facilitated by attaching a peristaltic pump to the autoloader, thus in the pumping mode mixing the sample with the OPA—3-MPA reagent in a mixing T-piece.

Within the complete HPLC system the autoinjector is connected to the controller by an interface cable. Full control of run time and gradient profile for each individual injection, as well as the number of repetitive injections from each individual vial, is then achieved.

RESULTS AND DISCUSSION

In previous reports, the concentration of free TML in human plasma has been estimated to ca. 0.1—0.5 μM [9, 20]. This minute concentration is far below the normal detection limit of a conventional amino acid analyser with ninhydrin detection. Thus, large amounts of plasma had to be enriched in TML prior to analysis. Furthermore, owing to the similar chromatographic properties of TML and lysine, a satisfactory separation of these amino acids required lengthy analysis times.

Recent developments in HPLC technology using fluorometric detection of amino acid adducts provide the basic prerequisites for appropriate analysis of TML because of the high speed and sensitivity of these methods [21, 22].

In the present work the main efforts were directed to achieve a higher sensitivity than that obtained by Davis et al. [16], thus allowing free TML measurement in an acceptable volume of human plasma. In Fig. 1 a typical chromatogram of a standard mixture of TML, TEL (internal standard) and Arg is depicted; this shows a satisfactory separation of these substances. Apparently pre-column derivatization, as used in the present study, leads to better resolution than observed with post-column derivatization [16]. Davis et al. [16] reported a detection limit of 25 pmol per injection, which enabled a satisfactory determination of plasma free TML concentrations in experimental rats. Bearing in mind, however, that free TML is present in rat plasma in concentrations ten times greater than in human plasma, their method is of little use in clinical practice because ca. 100 ml of human blood might have to be processed prior to HPLC analysis.

With the present experimental conditions we were able to achieve a detection limit for free TML of 3.5 pmol injected. Considering the concentrating factors throughout the sample preparation procedure (see Experimental) this would correspond to a final plasma concentration of 25 nM, and hence a suitable sample volume would be 10 ml, well within the reach of most clinical studies.

The fluorimetric detection of free TML is found to be linear up to 8.0 μM. When the TML peak area is expressed in arbitrary units, the concentration relationship is expressed by the equation $y = 0 + 15.1 \cdot [\text{TML}]$ (μM), $r = 0.998$ ($P < 0.001$). It is necessary to cover this large range when analysing the tenfold concentrated human plasma (see Experimental). Preliminary studies in dialysis patients revealed free plasma TML concentrations that exceed 1000 nM before dialysis [23]. Therefore the preparation of a smaller plasma volume is recommended in such pathological conditions.

By using the automated sample injector the reproducibility of the analytical system was determined in twelve consecutive standard runs to yield a coefficient of variation of 6.1%.

Under the experimental conditions described here free TML was determined

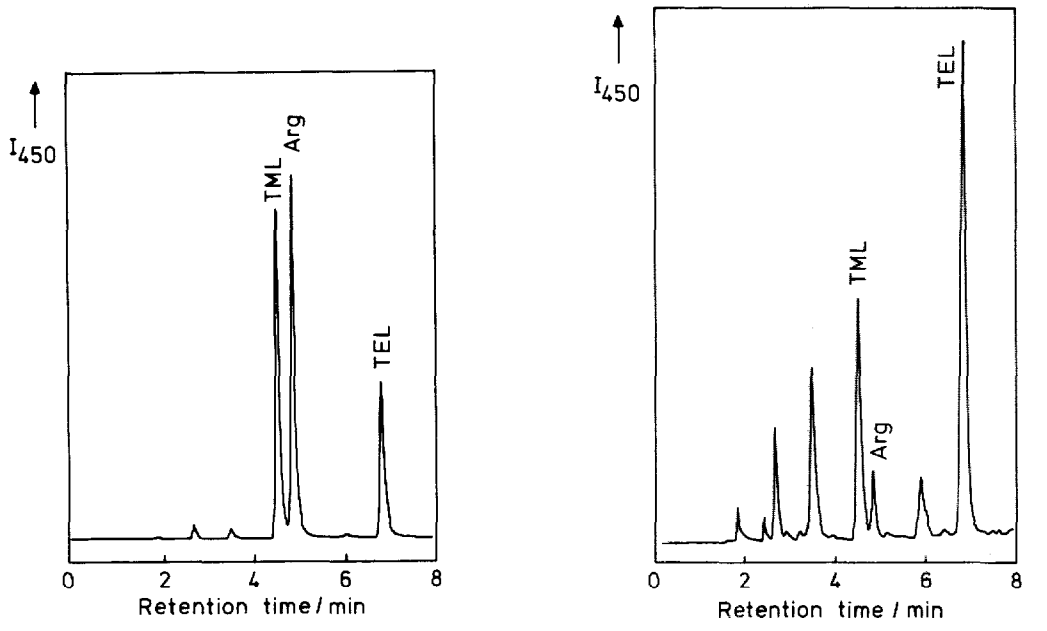

Fig. 1. Elution profile of OPA-derivatized standard mixture containing 2.5 μmol/ml each of trimethyllysine (TML), triethyllysine (TEL) and arginine (Arg).

Fig. 2. HPLC elution profile of human plasma after sample preparation and OPA derivatization (see Experimental). Peaks: TML = trimethyllysine; TEL = triethyllysine; Arg = arginine.

in human plasma (Fig. 2). As mentioned above, the great excess of the other free amino acids implies the need for a prior isolation of free TML in order to prevent overloading of the HPLC column. Fig. 2 indicates that free TML is well resolved from other "contaminating" amino acids. Especially important is the satisfactory separation of free TML from free Arg, because these two amino acids exhibit similar chromatographic properties under the experimental conditions. According to the report from Davis et al. [16], Arg is almost quantitatively removed, whereas in the present work the Arg peak is still eluted in measurable amounts. The fact that the sample preparation preserves a detectable Arg peak in human plasma, in contrast to the missing Arg peak in rat plasma despite the similar preparation procedure, might possibly be attributed to the considerable higher concentration ratio of Arg to TML in human plasma.

Efforts to reduce further the Arg contamination resulted in a decrease of TML recovery in the isolation procedure. Recovery of TML in each individual sample was feasible by considering the internal standard TEL, added to the samples prior to deproteinization.

Plasma TML concentrations were measured in twelve healthy males and eight females (age range 24—56 years). The mean value in the combined material was 290 ± 164 n*M*. However, an examination of the combined data revealed a significant sex difference, males exhibiting higher TML levels (355 ± 174 n*M*) than females (194 ± 87 n*M*, $P < 0.05$). This difference might be a reflection of the higher muscle mass present in males since the major reservoir of TML is represented by the skeletal muscle.

The data for human plasma free TML concentration presented in this report

confirm the early observations of Kakimoto and Akazawa [9], who measured 100 n*M* TML in one person, and those of Lange et al. [20], who observed in three individuals a mean plasma concentration of 420 n*M* (330—510 n*M*).

CONCLUSION

The development of the HPLC method described here should facilitate routine determination of plasma free TML in patients with suspected alterations in carnitine metabolism.

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