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Note

Gas chromatographic—mass spectrometric characterization of N-methylated basic amino acids in human urine

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Methylamino acids such as 3-methylhistidine (3-MeHis), N^{ϵ}-monomethyllysine (MML), $N^{\epsilon},N^{\epsilon}$-dimethyllysine (DML), $N^{\epsilon},N^{\epsilon},N^{\epsilon}$-trimethyllysine (TML), N^{G},N^{G}-dimethylarginine (N^{G},N^{G}-DMA) and N^{G},N'^{G}-dimethylarginine (N^{G},N'^{G}-DMA) are normal constituents of human urine [1]. These are also the constituents of several tissue proteins of various organs [2]. Specific sites of histidine, lysine and arginine residues of the proteins are methylated after protein synthesis. The methylated amino acids are released upon protein degradation and excreted into urine without being reutilized for protein synthesis [3]. Muscle and connective tissue proteins constitute the predominant proportions of the structural proteins of the animal body. Connective tissue proteins are not methylated. Of muscle proteins, actin contains 3-MeHis residue [4], and myosin contains 3-MeHis, DML, TML and N^{G},N^{G}-DMA residues [5]. 3-MeHis in urine originates only from these two proteins [2, 6, 7] and large amounts of DML, TML and N^{G},N^{G}-DMA in urine originate from muscle proteins.

Any gross change in the amounts or turnover rates of the muscle proteins may therefore be reflected in a change in the amounts of methylamino acids in urine in the presence of pathological changes of muscle tissue. Accurate determination of the concentrations of methylated amino acids in biological samples is essential because their concentrations in some instances are very low, but no specific assay has been reported. Methylated amino acids have been determined mostly by ion-exchange chromatography and a number of methods have been published specifically for their analysis [2, 8—12]. Recently we developed a method for the isolation of 3-MeHis from biological specimens and its characterization by glass capillary gas chromatography (GC) [13].

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This present report describes a method for the derivatization and detection of N^G-MMA, N^G,N^G-DMA, N^G,N'^G-DMA, MML, DML and TML using a glass capillary GC technique and gas chromatography—mass spectrometry (GC—MS).

EXPERIMENTAL

Materials

N^G-Monomethyl-L-arginine (N^G-MMA) [di-(*p*-hydroxyazobenzene-*p'*-sulphonate)], N^G,N^G-dimethyl-L-arginine [di-(*p*-hydroxyazobenzene-*p'*-sulphonate)], N^G,N'^G-dimethyl-L-arginine [di-(*p*-hydroxyazobenzene-*p'*-sulphonate)], N-ε-methyl-L-lysine · HCl and N-ε-trimethyl-L-lysine, bis(*p*-hydroxyazobenzene-*p'*-sulphonate) were obtained from Calbiochem-Behring (La Jolla, CA, U.S.A.). N-ε-Dimethyl-L-lysine · HCl was synthesized according to the method described by Benoiton [14]. The arginine and lysine bis(*p*-hydroxyazobenzene-*p'*-sulphonate) were converted to the free base form after esterification, as reported below, and MML and DML crystallized as hydrochlorides were used as such.

Derivatization

The preparation of methylarginine and methyllysine derivatives is a two-step process, initially requiring esterification of the carboxyl groups. As esterification agent, the mixture of dry acetyl chloride 5% in *n*-propanol was chosen; 5 ml of the esterification mixture were added to 0.2 μmol of MML, DML and 25 mg of TML, N^G-MMA, N^G,N^G-DMA and N^G,N'^G-DMA. Each vial was sealed, mixed and left to react overnight at 90° C. Samples were evaporated to dryness under vacuum, then MML and DML were N-acylated with 250 μl of trifluoroacetic anhydride and 750 μl of ethyl acetate in sealed vials at 110° C for 30 min.

In order to remove *p*-hydroxyazobenzene-*p'*-sulphonic acid, N^G-MMA, N^G,N^G-DMA, N^G,N'^G-DMA and TML propyl ester, each dissolved in 0.2 ml of *n*-propanol were passed through a column (7 × 1 cm) of silica gel 60 extra pure (70—230 mesh ASTM) (E. Merck, Darmstadt, F.R.G.) packed with chloroform and equilibrated with 20 ml of a mixture of *n*-propanol—acetic acid— water (4:1:1). The column was eluted with 25 ml of the equilibration mixture; the first 15 ml of intensely yellow effluent, containing *p*-hydroxyazobenzene-*p'*-sulphonic acid, were discarded and the next 10 ml of effluent were evaporated to dryness under nitrogen. TML, N^G-MMA, N^G,N^G-DMA and N^G,N'^G-DMA propyl ester in amounts of 0.2 μmol each were subsequently trifluoroacetylated as previously described. The completeness of the derivatization steps was checked by thin-layer chromatography. After evaporation, the residue was dissolved in 1 ml of ethyl acetate to give a final concentration of 0.2 m*M* of each amino acid; 1 μl was sufficient for analysis (0.2 nmol).

Apparatus and instrumental conditions

N^G-MMA, N^G,N^G-DMA, N^G,N'^G-DMA, MML, DML and TML derivatives give sharp, symmetrical peaks on common stationary phases such as pretested QF-1, SE-30, OV-1, OV-17 and OV-101 (Applied Science Labs., State College, PA, U.S.A.) in packed columns, but when biological samples are analysed,

resolution is not as good as that of the standard pool because of interfering peaks. For this reason glass capillary columns were chosen.

A high-resolution dedicated gas chromatograph 3900-B (Dani, Monza, Italy) equipped with a flame ionization detector was used. The glass capillary column (20 m × 0.85 mm O.D., 0.30 mm I.D., Duran 50) was prepared according to the barium carbonate procedure described by Grob et al. [15] and given a 0.15-μm thick Pluronic F-68 coat using the static procedure. The split injection mode was used. Temperatures were as follows: oven programmed from 60 to 200°C, detector 280°C, injector 300°C. Carrier gas was hydrogen (oxygen-free) with a flow-rate of 0.7 ml/min. Splitter flow was 15 ml/min. The chromatograph was equipped with an Infotronic CR S-124 digital integrator for determination of peak areas for quantitative work. For quantitative studies, internal standardization with 1,2,4,5-tetramethylbenzene (TMB) (B.D.H., Poole, U.K.) dissolved in ethyl acetate (0.2 m*M*) was used.

RESULTS AND DISCUSSION

A typical gas chromatogram of synthetic N^G-MMA, N^G,N^G-DMA, N^G,N'^G-DMA, MML, DML and TML derivatives is illustrated in Fig. 1. The separation is quite good and preliminary experiments using the isolation procedure of Kakimoto and Azakawa [1] from rat and human urine and the derivatization procedure described for synthetic substances indicated there were no interfering peaks in these biological samples (Fig. 2).

Briefly the isolation procedure was carried out as follows: an amount of urine containing 10 mg of creatinine was passed through a column (0.9 × 5 cm)

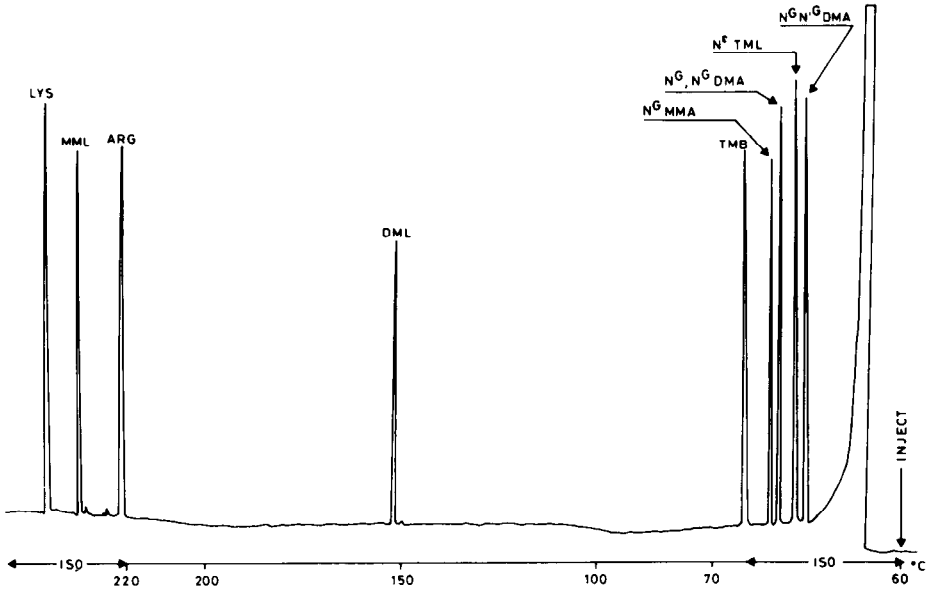

Fig. 1. Typical chromatogram of synthetic methylated basic amino acid derivatives. A Duran 50 glass capillary column (20 m × 0.85 mm O.D., 0.30 mm I.D.) was used with a 0.15-μm thick Pluronic F-68 coat. The split injection mode was used. Injector temperature was 220°C. Flame ionization detector was set to 250°C. Oven temperature programme: 60°C for 5 min, raised at 5°C/min to 220°C, held constant for 5 min.

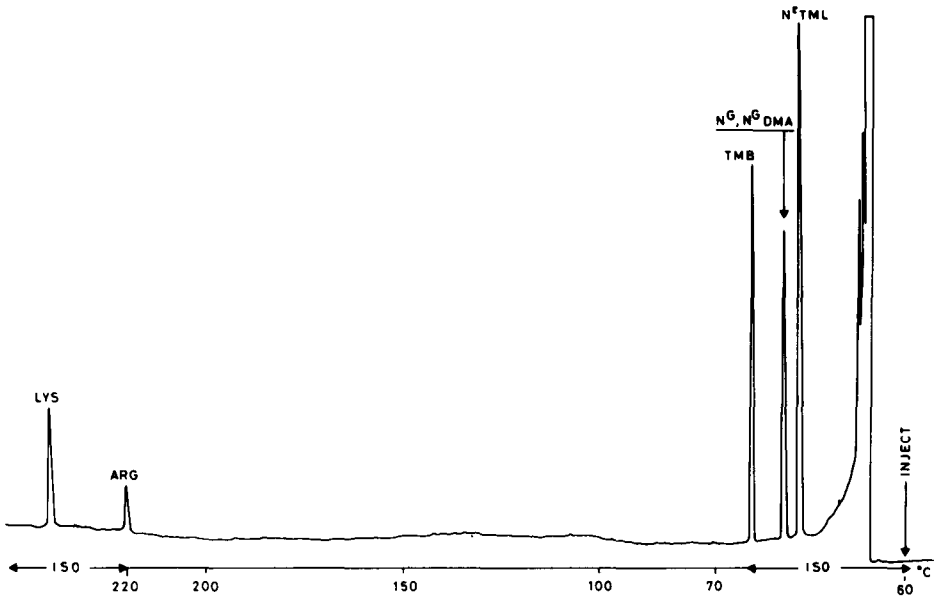

Fig. 2. Typical chromatogram obtained from the assay of urinary human methylated basic amino acids measured in μmol/g creatinine. N^eTML = 55, N^G,N^G-DMA = 49. GLC conditions for separation as in Fig. 1.

of AG 50 W × 4 resin, H^+ form, 100—200 mesh (Bio-Rad Labs., Richmond, CA, U.S.A.). The column was washed with 20 ml each of water and 1 *M* pyridine, and the adsorbed basic amino acids were eluted with 20 ml of 3 *M* ammonium hydroxide. The eluate was evaporated to dryness under vacuum. The residue was dissolved in 2 ml of water and the solution was passed through a column (0.9 × 5 cm) of Bio-Rex 70 resin NH_4^+ form, 100—200 mesh (Bio-Rad Labs.). The resin was washed with 20 ml of water, and the retained amino acids were eluted with 20 ml of 2 *M* ammonium hydroxide. The eluate was evaporated to dryness under vacuum and the residue was submitted to the derivatization procedure described for synthetic substances.

Recovery of these methylated basic amino acids added to human and rat urine in amounts of 100 μg each was always 90—95% (S.D. = 3%). The limit of sensitivity of the method was 2.0 nmol/ml. The reproducibility was determined by repeated analyses of the synthetic mixture of the six methylated basic amino acids. The intra-assay coefficient of variation, calculated from ten chromatograms in sequence, was 0.10% . The inter-assay coefficient of variation (n = 10) was 0.15%. For each of the methylated basic amino acids the sample concentration injected was plotted against the ratio between the area of each amino acid and that of the internal standard. With concentrations up to 10 pmol/ml of each amino acid a linear relation (regression coefficient = 0.99) was found. The identity of GC peaks of the methylated basic amino acids tested was elucidated by GC—MS (Figs. 3—5).

With the exception of TML, all the derivatives tested gave fragmentation patterns consistent with the structure of propyltrifluoroacetyl derivatives (mass spectra of methylarginines are unreported because quite expected). Figs. 3 and 4 show the mass spectra of MML and DML. Both show a very

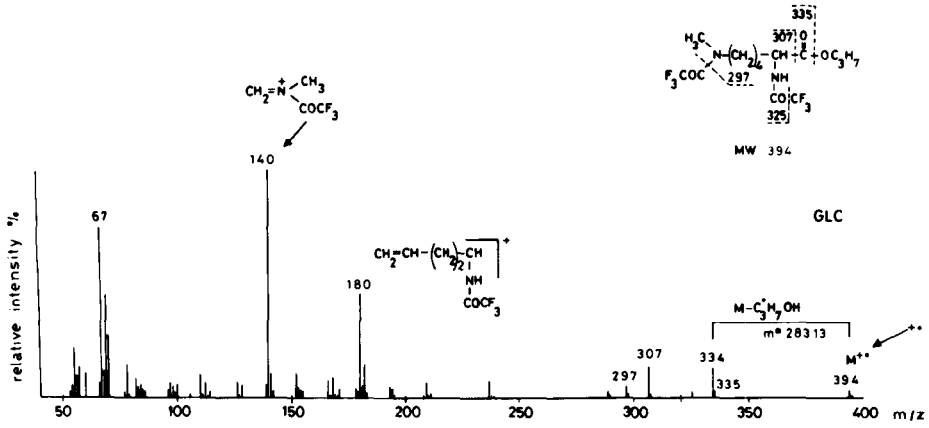

Fig. 3. Mass spectrum of MML. GLC—MS conditions and fragment ions as given in the text.

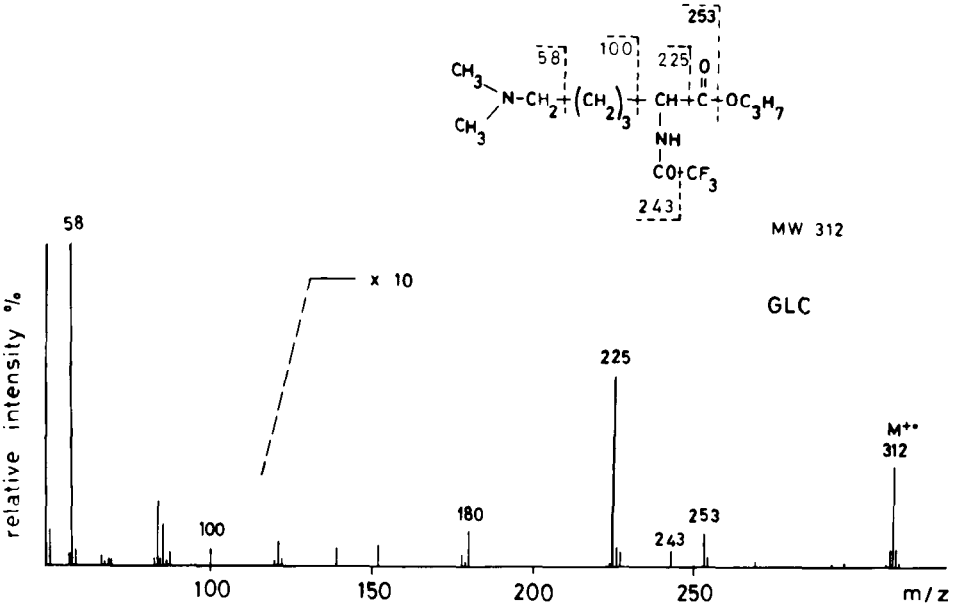

Fig. 4. Mass spectrum of DML. GLC—MS conditions and fragment ions as given in text.

intense peak at *m/z* 180 and the molecular ions are *m/z* 394 and 312, respectively. The mass spectrum of TML is shown in Fig. 5. The spectrum consists chiefly of one very intense peak at *m/z* 180, common to all the methyllysine derivatives tested. The molecular ion at *m/z* 267 is an artefact probably resulting from the extremely easy thermal degradation of TML; in fact the molecular weight of the TML derivative is 327. At the bottom of Fig. 5, the mass spectrum analysed by direct inlet system MS shows the molecular ion at *m/z* 312, indicating thermal degradation of the TML derivative. For this reason, gas—liquid chromatography (GLC) of the TML derivative gives no warranty of reproducible results and further studies are required to quantify the thermal phenomenon. In order to make easier quantitative GLC determination of those methylated basic amino acids at present available as

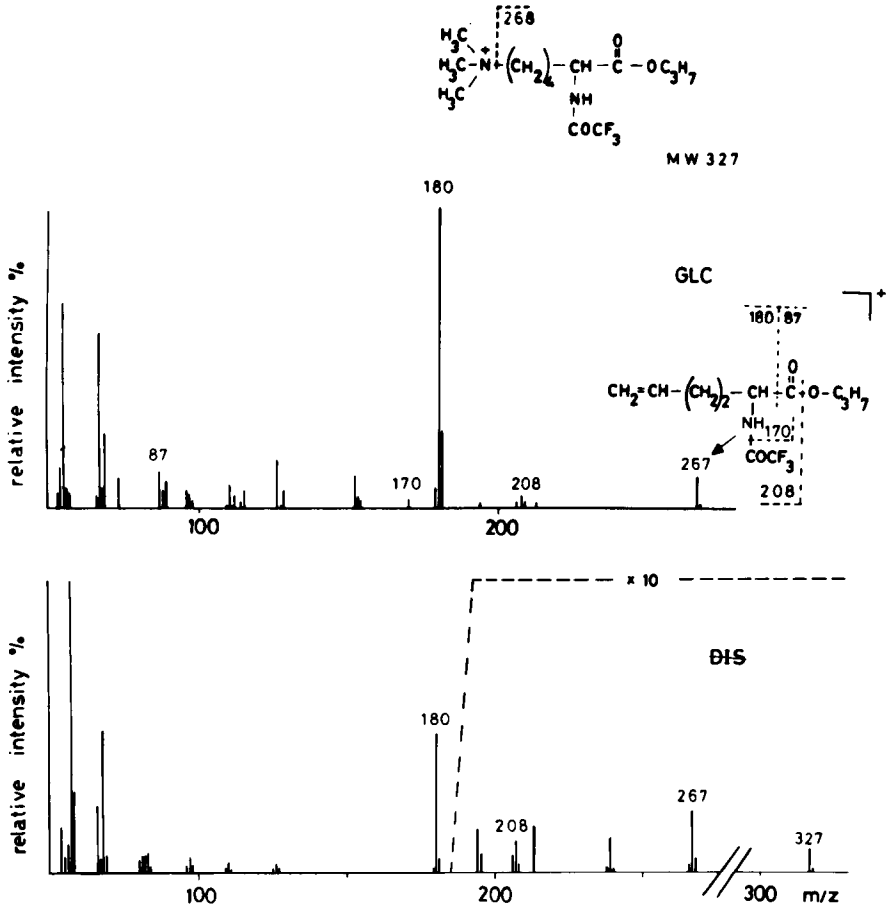

Fig. 5. Mass spectrum of TML. GLC—MS conditions and fragment ions as given in text. At the bottom mass spectrum analysed by direct inlet system MS.

di-(*p*-hydroxyazobenzene-*p'*-sulphonate) salts, we are preparing them according to the methods of Benoiton [14] and Kakimoto and Azakawa [1].

The present method could be useful for the quantitative determination of methylated basic amino acids in human urine in conditions involving pathological changes of muscle tissue.

ACKNOWLEDGEMENTS

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