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Note

Glass capillary quantitative determination of N$^{\pi}$-methylhistidine in urine and muscles

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There are a number of methylated amino acid derivatives in muscle, including N$^{\tau}$-methylhistidine (3-methylhistidine) (3-MeHis), N$^{\pi}$-methylhistidine (1-methylhistidine) (1-MeHis) [1], the mono- and trimethyllysines [2—4] and N^{G},N^{G}-dimethylarginines [5, 6]. The function of such methylated amino acids is not clearly understood, but it has been proposed that the methyllysines and methylarginines of histones might be involved in the regulation of DNA synthesis and chromatin activity [7]. Furthermore, it has been found that trimethyllysine is the precursor of 4-trimethylaminobutyrate for carnitine biosynthesis [8, 9]. However, neither the function of 3-MeHis residue in actin and myosin, nor the significance of 1-MeHis in the dipeptide anserine has been established. It appears that 1-MeHis is not made by methylation of histidine in proteins.

Accurate measurement of the concentration of methylated amino acids in biological samples is essential because their amount in some instances is very low and no specific assay has been reported. Most workers have determined some methylated amino acids by ion-exchange chromatography and a number of methods have been published specifically for the analysis of methyl amino acids [6, 10—14].

Recently we have developed a method for the isolation of 3-MeHis from biological specimens and its quantitative determination by glass capillary gas chromatography (GC) [15]. This paper describes the application of such a method for the isolation from biological samples and quantitative determination of 1-MeHis.

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EXPERIMENTAL

Isolation of 1-MeHis by charcoal column chromatography

The analytical procedure is substantially the same as that used for the isolation of 3-MeHis from biological samples. It can be summarized as follows. Silanized columns (1.5 × 1 cm I.D.) were packed with charcoal—celite (1:1, w/w) (BDH, Poole, Great Britain) suspended in and washed with 1 *N* HCl. Subsequently the columns were washed with 20 ml of water and buffered at pH 5 with 0.33 *M* acetate buffer (5 ml). Biological samples (hydrolyzates of urine and muscles evaporated to dryness under vacuum) were dissolved in 0.33 *M* acetate buffer (pH 5) and an amount corresponding to 0.25 ml of urine and 15 mg of wet muscle was applied to the charcoal—Celite column which was washed first with 20 ml of water, then 5 ml of 80% acetone; 1-MeHis was eluted with 30 ml of dichloromethane—methanol—33% ammonium hydroxide (70:25:5). The eluates were evaporated to dryness under vacuum.

Derivatization of 1-MeHis for gas chromatography

Preparation of the 1-MeHis derivative is a two-step process, initially requiring that the carboxyl group be esterified. Among esterification agents, a mixture of dry acetyl chloride 5% in propanol was chosen; 5 ml of the esterification mixture were added to the residue. Each tube was sealed, mixed and left to react overnight at 90°C in a Reacti—Therm-Heating module (Pierce, Rockford, IL, U.S.A.). Samples were evaporated to dryness under vacuum and then N-acetylated with 150 μl of trifluoroacetic anhydride and 200 μl of dichloromethane for 30 min at room temperature. The 1-MeHis derivative was evaporated to dryness under vacuum and redissolved in dichloromethane solution containing the reference standard (*n*-triacontane 50 μg/ml) before GC analysis. 1-MeHis was completely derivatized (propyl ester, N-trifluoroacetate) as checked by thin-layer chromatography.

Chromatographic conditions

The 1-MeHis derivative gives sharp, symmetrical peaks on common stationary phases such as pretested SE-30, OV-1, OV-17, OV-101 (Applied Science Labs., State College, PA, U.S.A.) in packed columns, but there is no resolution between 1- and 3-MeHis peaks. For this reason we preferred to work with glass capillary columns.

The gas chromatograph was a high-resolution dedicated gas chromatograph 3900B (Dani, Monza, Italy) equipped with a flame ionization detector. The glass capillary column (20 m long, 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. [16] and given a 0.15 μm thick Pluronic F-68 coating using the static procedure. The split injection mode was used. Temperatures were over 200°C, detector 280°C and injector 300°C. Carrier gas was hydrogen (O_2-free) with a flow-rate of 0.7 ml/min. Splitter flow was 15 ml/min. The practicable sensitivity limit was about 2 μg/ml. The use of an electron-capture detector increased the sensitivity about 100-fold but this detector was not routinely used because rat urine contains a large amount of 1-MeHis. The electron-

capture detector should be very useful for measuring low concentrations of 1-MeHis as, for example, in muscle proteins.

Mass spectrometric assays were performed on an LKB-9000 (Bromma, Sweden) interfaced with a 3% OV-1 packed column.

Quantitation

Quantitation was performed using the method of internal standardization with *n*-triacontane. The calibration curve for 1-MeHis derivative concentrations ranging from 12.5 to 100 ng/μl showed a linear response within this range of concentrations.

Urine creatinine was determined on urine samples using 3,5-dinitrobenzoic acid, according to the instructions supplied with Eurochima Kit (Elvi, Milan, Italy). Protein was determined by the method of Lowry et al. [17].

RESULTS AND DISCUSSION

A typical gas chromatogram of rat urine is illustrated in Fig. 1, where 1-MeHis is present at a greater concentration than 3-MeHis. Recoveries of 1-MeHis from rat urine were 96 ± 2%, the mean ± S.E.M. of four determinations. No significant differences were found between recoveries for different 1-MeHis concentrations (25, 50, 75, 100 ng).

Mass spectrometric analysis confirmed that the 1-MeHis derivative is a

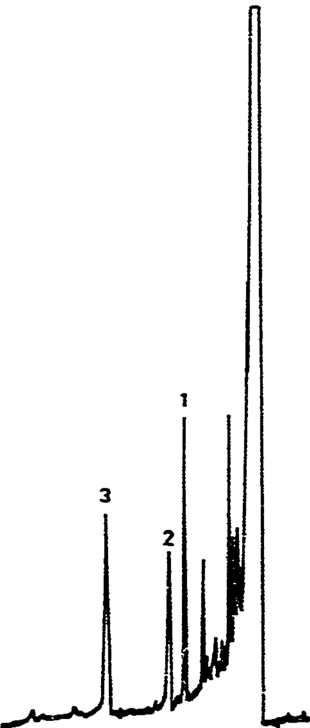

Fig. 1. Gas chromatogram of rat urine sample. 1 = 3-MeHis derivative; 2 = 1-MeHis derivative; 3 = reference standard (*n*-triacontane, 50 μg/ml).

propyl ester, N-trifluoroacetate, as shown in Fig. 2. The basal levels of 1-MeHis and 3-MeHis in rat urine are reported in Table I. Results of preliminary studies of 1-MeHis and 3-MeHis concentrations in rat muscle are reported in Table II.

Chromatographic peaks of biological samples were identified with precision by comparison of the mass spectra of the biological compound and the authentic reference standard, obtained by electron impact.

Critical factors during derivative formation are that reagents must be free of any trace of moisture, and that the preceding reagent must be completely

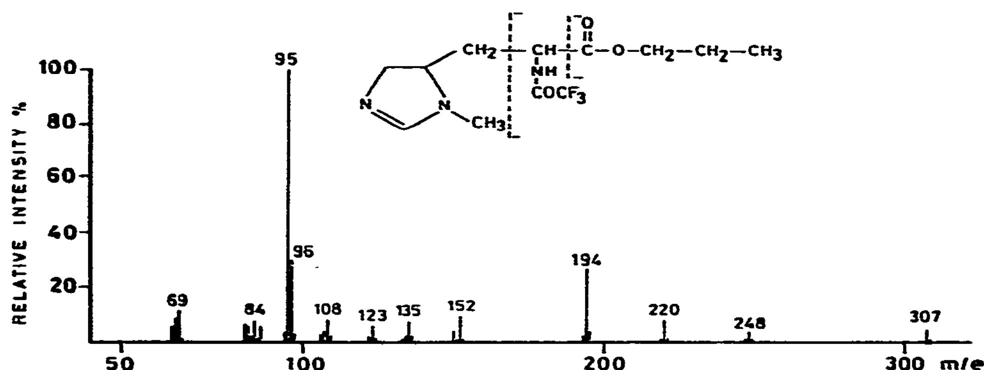

Fig. 2. Mass spectrum of 1-MeHis derivative.

TABLE I

DAILY EXCRETION OF CREATININE, 1-MeHis AND 3-MeHis BY MAN AND BY ADULT MALE RATS

	Creatinine excretion (mmol·24 h^{-1})	1-MeHis excretion (μmol·24 h^{-1})	3-MeHis excretion (μmol·24 h^{-1})
Man*			
C.A.	7.31	958.30	184.80
P.L.	5.34	741.90	151.10
R.M.	3.50	561.08	112.29
R.F.	5.82	920.40	189.17
Mean	5.49 ± 0.95	795.42 ± 99.30	159.34 ± 19.72
Rats**			
1	0.043	2.50	1.64
2	0.057	3.53	2.41
3	0.060	3.15	2.02
4	0.069	3.45	2.31
5	0.079	3.52	2.21
Mean	0.061 ± 0.007	3.03 ± 0.20	2.12 ± 0.15

*Healthy boys (7—12 years), weight 16—25 kg.
**Male CD-COBS rats (Charles River, Calco, Italy), body wt. 250 g.

TABLE II

CONCENTRATIONS OF 1-MeHis AND 3-MeHis IN SOME RAT MUSCLES

Male CD-COBS rats (Charles River), body wt. 250 g, were used. Muscles were pooled from two animals for each determination. Values are expressed as mean ± S.E.M. (n = 4).

Muscle	Protein in wet muscle (mg/g)	Muscle protein (μg/g)	
		1-MeHis	3-MeHis
Palmaris longus	72.96 ± 2	3111 ± 10	886 ± 9
Pectoralis	85.76 ± 3	1762 ± 14	660 ± 10
Gastrocnemius	98.40 ± 5	1730 ± 12	774 ± 8
Heart	103.12 ± 7	543 ± 6	434 ± 6

eliminated by evaporation before the next derivatization step.

The present method, with a sensitivity of 2 μg/ml, appears reliable for measuring 1-MeHis and 2-MeHis in the same sample of urine from animals or men in normal and pathological conditions. The use of an electron-capture detector increases the sensitivity about 100-fold, thus making the method suitable for measuring very low levels of these methylhistidines, in studies of the catabolic rates of muscle proteins, for instance.

Further studies are in progress to measure muscle methylhistidine turnover in animals and men.

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