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Note

Glass capillary gas chromatographic determination of N^{τ}-methylhistidine in urine

L. COTELLESSA, F. MARCUCCI*, D. CANI, P. SFONDRINI, L. COLOMBO and E. MUSSINI

Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan (Italy)

and

F. POY *Dani S.p.A., Monza (Italy)*

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Since the potential utility of measuring urinary N^{τ}-methylhistidine [His-(τMe)] for determining the catabolic rate of skeletal muscle protein was first suggested [1], confirmation has been provided by several workers [2—6]. Catabolic rates of muscle proteins under various nutritional conditions have been estimated by this method [7—10] and recently the use of His(τMe) excretion as an index of myofibrillar protein breakdown was reviewed [11].

Accurate determination of the concentration of His(τMe) in muscle protein is essential because its concentration is very low, but no specific assay for His (τMe) has been reported. Most workers have determined His(τMe) by ion-exchange chromatography and a number of methods have been published specifically for the analysis of methylamino acids [3,12—16].

This paper describes a method for the isolation from biological specimens and quantitative determination of His(τMe) by glass capillary gas chromatography (GC).

EXPERIMENTAL

Isolation of His(τMe) by ion-exchange column chromatography (Method A)

Ion-exchange chromatography was performed using 200—400 mesh AG 50W-X8 (Bio-Rad Labs., Richmond, CA, U.S.A.) as described by Ward [17]. The resin was packed by gravity into a column (5 × 1 cm I.D.) to give a bed height of 3.5 cm. The column had previously been equilibrated with 0.2 *M* pyridine (20 ml). Since some His(τMe) is excreted in the N-acylated form [2],

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urine was hydrolyzed before analysis by heating in an equal volume of 12 *N* HCl at 110°C overnight in sealed vials. The hydrolyzates were evaporated to dryness under vacuum and the residues were dissolved in 0.2 *M* pyridine and applied to the column in a volume of 2 ml (corresponding to 0.25 ml of urine). The acid and neutral amino acids were eluted with 20 ml of 0.2 *M* pyridine. His(τMe) was then eluted with 10 ml of 1 *M* pyridine. The eluent flow-rate was 10 ml/h. The His(τMe) fraction (also containing 1-methylhistidine and histidine) thus obtained was evaporated to dryness under vacuum. The dried samples were then derivatized to make them suitable for GC analysis.

Isolation of His(τMe) by charcoal column chromatography (Method B)

Column adsorption chromatography was performed using charcoal—Celite (1:1, w/w) (BDH, Poole, Great Britain) suspended and washed with 1 *N* HCl. Columns (1.5 × 1 cm I.D.) were silanized with 10% Dri Film (Pierce, Rockford, IL, U.S.A.) in carbon tetrachloride, then washed with 20 ml of water and buffered at pH 5 with 0.33 *M* acetate buffer (5 ml). The hydrolyzates were evaporated to dryness under vacuum and the residues were dissolved in 0.33 *M* acetate buffer (pH 5) and an amount corresponding to 0.25 ml of urine was applied to the charcoal—Celite column. After washing with 20 ml of water followed by 5 ml of 80% acetone, His(τMe) was eluted with 30 ml of dichloromethane—methanol—33% ammonium hydroxide (70:25:5). The eluates were evaporated to dryness under vacuum.

This second analytical procedure was routinely used for His(τMe) determination in human and rat urine, because of its speed, ease and simplicity.

Derivatization of His(τMe) for gas chromatography

The preparation of the His(τMe) derivative is a two-step process, initially requiring that the carboxyl group be esterified. From among the esterification agents the mixture of dry acetyl chloride 5% in propanol was chosen; 5 ml of the esterification mixture were added to the residue. Each tube was capped, mixed and left to react overnight at room temperature. Samples were evaporated to dryness under vacuum, then N-acylated with 150 μl of trifluoroacetic anhydride and 200 μl of methylene dichloride for 30 min at room temperature. The His(τMe) derivative was evaporated to dryness under vacuum and redissolved in a methylene dichloride solution of the reference standard (*n*-triacontane 50 μg/ml) before GC analysis. His(τMe) was completely derivatized as the propyl ester, N-trifluoroacetate, checked by thin-layer chromatography.

GC analytical conditions

The His(τMe) derivative gives sharp and 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 when biological samples are analyzed, resolution is not quite as good as that of the standard pool because of interfering peaks. For this reason it was preferred to work with glass capillary columns.

The gas chromatograph was a high-resolution dedicated gas chromatograph 3900-B (Dani, Monza, Italy) equipped with a flame-ionization detector. 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. [18] 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 200°C, detector 280°C, 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 mass-spectrometric assays were done on an LKB-9000 (Bromma, Sweden) interfaced with a 3% OV-1 packed column.

The practicable sensitivity limit is 12 nmol/ml. The use of an electron-capture detector (ECD) increased the sensitivity about 100-fold, but this detector was not needed because human and rat urine contain large amounts of His-(τMe). The ECD should be very useful for measuring low concentrations of His(τMe), in muscle protein, for example.

Quantitation

This important step was performed using the method of internal standardization with *n*-triacontane. The calibration curves for His(τMe) derivative concentrations ranging from 2.5 to 100 ng/μl indicated a linearity in the response within this range of concentrations.

Urinary creatinine determinations were performed on samples of the urine using 3,5-dinitrobenzoic acid according to the instructions supplied with Eurochima Kit (Elvi-Milano, Milan, Italy).

RESULTS

A typical gas chromatogram of rat urine samples is illustrated in Fig. 1.

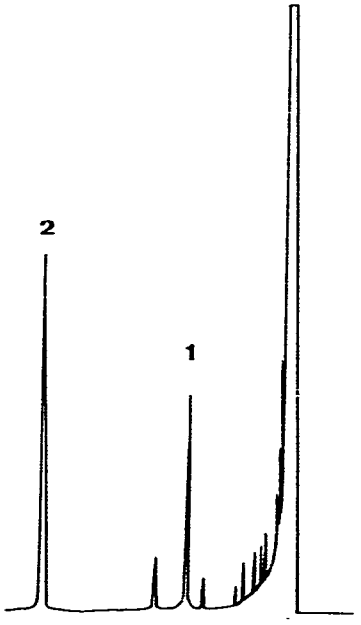

Fig. 1. Gas chromatogram of human urine sample. 1 = His(τMe) derivative; 2 = reference standard (*n*-triacontane 50 μg/ml).

TABLE I

EXCRETION OF TOTAL HIS(τMe) BY YOUNG HEALTHY ADULT MEN AND BY ADULT MALE RATS

	Body weight (kg)	Creatinine excretion (mg/ml)	His(τMe) excretion (μg/ml)	Ratio His(τMe)/creatinine (μg/ml)
Humans				
M.C.	53	1.84	30.35	16.49
E.Z.	63	1.42	23.84	16.78
M.S.	79	2.93	49.10	16.75
P.D.P.	66	2.64	41.44	15.69
Mean		2.20±0.3	36.18±6.0	16.40±0.2
Rats*				
1	—	0.34	14.10	41.22
2	—	0.57	21.45	41.73
3	—	0.66	29.35	44.00
4	—	0.58	24.05	41.11
Mean		0.54±0.08	22.26±3.81	42.01±0.72

*Male CD-COBS rats (Charles River, Italy) of body wt. 250 g were used.

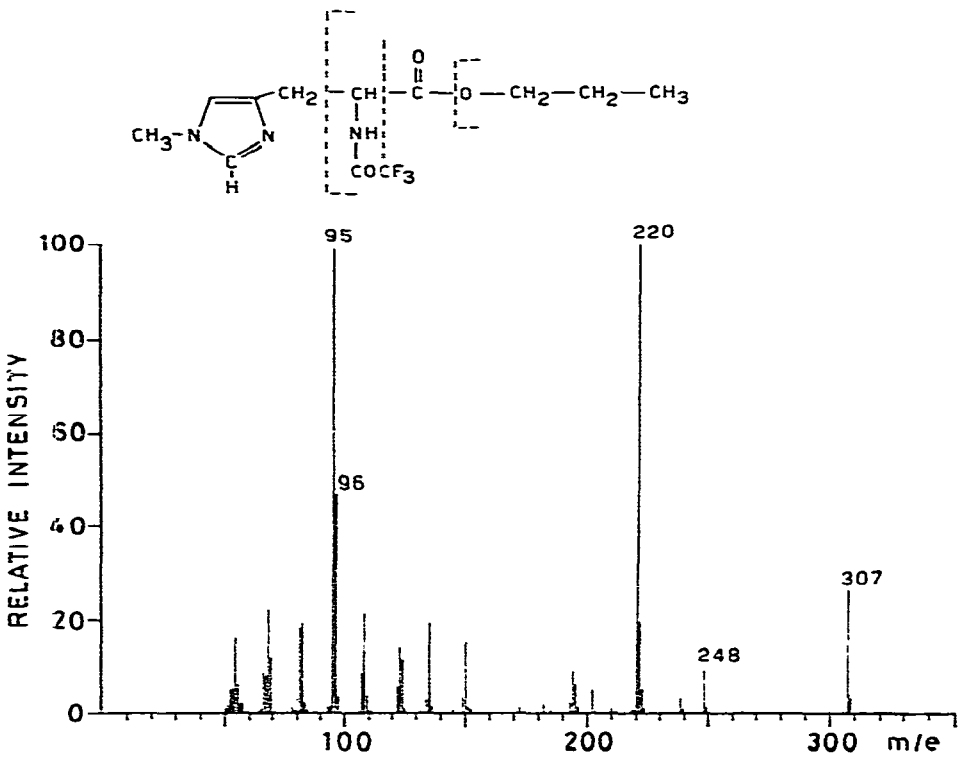

Fig. 2. Mass spectrum of His(τMe) derivative.

Human samples give a similar GC trace. Recoveries for His(τMe) from human and rat urine were, respectively, 99±1% and 98±1%, the mean ± S.E.M. of four determinations. No significant differences were found between recoveries for different His(τMe) concentrations (25, 50, and 100 μg). The basal levels of His(τMe) in human and rat urine are reported in Table I.

Mass spectrometric analysis confirmed that the His(τMe) derivative is a propyl ester, N-trifluoroacetate, as shown in Fig. 2.

DISCUSSION

During the extraction step, 1-methylhistidine and histidine are eluted together with His(τMe). Subsequently they are derivatized with His(τMe), but, whereas 1-methylhistidine is easily derivatized, the histidine derivative is very difficult to prepare because of the presence of both the monoacyl and diacyl derivatives. This difficulty has been reported by several workers [19—21]. For this reason our interest was restricted to His(τMe) analysis because otherwise the analytical procedure for biological samples becomes very complicated. Chromatographic peaks of biological samples were identified with precision by comparison of the mass spectra of the biological compound and of authentic reference standards obtained by electron impact. Critical factors during derivative formation are that the reagents must be free of any trace of moisture, and that the preceding reagent must be completely eliminated by evaporation before the next derivatization step is undertaken.

The present method, with a sensitivity of 5 ng/μl, appears to be reliable for determining His(τMe) in urine of animals and man in normal and pathological conditions. The use of an ECD increases the sensitivity about 100-fold, thus making it suitable for measuring very low His(τMe) levels, in studies of the catabolic rates of muscle proteins, for instance.

Further studies are in progress to measure muscle His(τMe) turnover in animals and man.

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