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Note

Gas chromatographic quantitative determination of 1- and 3-methylhistidine in urine and muscles: comparison with glass capillary determination

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Interest in the determination of the methylhistidine isomers, 1-methylhistidine (1-MH) and 3-methylhistidine (3-MH), has been increasing recently. 3-MH is present as a single residue in the peptide chains of actin and myosin [1, 2]. It is thus particularly abundant in muscle. 1-MH has been isolated in a peptide of the neuromuscular junction, whose function is still unknown. Upon breakdown of actin and myosin, 3-MH is not re-utilized, but is rapidly and quantitatively excreted in the urine [3, 4]. It is therefore a useful urinary marker of muscular turnover. The usual analysis techniques for these two MH isomers are based on ion-exchange chromatography with either the amino acid analyser [5] or a colorimetric method using ninhydrin and *o*-phthalaldehyde [6, 7]. High-performance liquid chromatography [8] or gas chromatography [9] can also be used.

Recently, we have developed a method for the isolation of the two MH isomers from biological specimens and their quantitative determination by glass capillary gas chromatography (GC) [10]. This method has been applied for the determination of 1- and 3-MH in various animal and human studies [11—13]. However, the glass capillary GC method requires sophisticated equipment and experienced maintenance staff, so it is not practical for routine clinical analysis.

The aim of the present study was to develop a simpler GC method using a normal GC apparatus and a packed column. The method was applied for the determination of 1- and 3-MH in human urine and rat muscles, and its capabilities were compared with the glass capillary GC method.

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EXPERIMENTAL

Isolation of 1- and 3-MH by charcoal column chromatography

Column adsorption chromatography was performed as previously described [10] using columns (1.5 × 1.0 cm I.D.) packed with charcoal—Celite (1:1, w/w) and buffered at pH 5 with 0.33 *M* acetate buffer. Dried hydrolysates of biological samples 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 first washed with 20 ml of water, then with 5 ml of 80% acetone; 1- and 3-MH were eluted with 30 ml of dichloromethane—methanol—33% ammonium hydroxide (70:25:5). The eluates were evaporated to dryness under vacuum.

Derivatization of 1- and 3-MH for gas chromatography

The carboxy group of 1- and 3-MH was esterified with 5 ml of a mixture of 5% dry acetyl chloride in *n*-propanol. 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 two derivatives were evaporated to dryness under vacuum and redissolved in ethyl acetate solution containing as reference standard 2-N-methylamino-5-chlorobenzophenone (MACB, 25 μg/ml) before GC analysis.

GC analytical conditions

A Fractovap 2150 gas chromatograph (Carlo Erba, Milan, Italy) equipped with a flame-ionization detector was used. The glass column (2 m × 4 mm I.D.) was silanized and packed with 1.5% SP-2250 and 1.95% SP-2401 on 100—120 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). The temperatures of the injection port, column and detector were kept at 275°C, 210°C and 275°C, respectively. Nitrogen was used as the carrier gas at a flow-rate of 33 ml/min. The practical sensitivity limit was about 4 μg/ml, compared to 2 μg/ml with the glass capillary GC method.

The use of an electron-capture detector increased the sensitivity about 100-fold but this detector was not routinely used because human urine contains large amounts of 1- and 3-MH. This detector should be very useful for measuring low concentrations of the two isomers as, for example, in muscle proteins.

Mass spectra of the two MH isomers have already been reported [10].

Quantitation

Quantitation was by the internal standardization method with MACB. The calibration curves for 1- and 3-MH derivative concentrations from 12.5 to 100 ng/μl showed a linear response within this range. Urinary creatinine was determined on urine samples using a modification of the alkaline picrate technique [14]. Proteins were determined by the method of Lowry et al. [15].

RESULTS AND DISCUSSION

A comparison of the resolution capacity of the packed column and the glass capillary column in the separation of a sample of human urine is shown in Fig. 1. The 1- and 3-MH derivatives were satisfactorily separated, with short retention times and symmetrical peaks in the packed GC analytical conditions described. No interfering peaks were detected in biological samples.

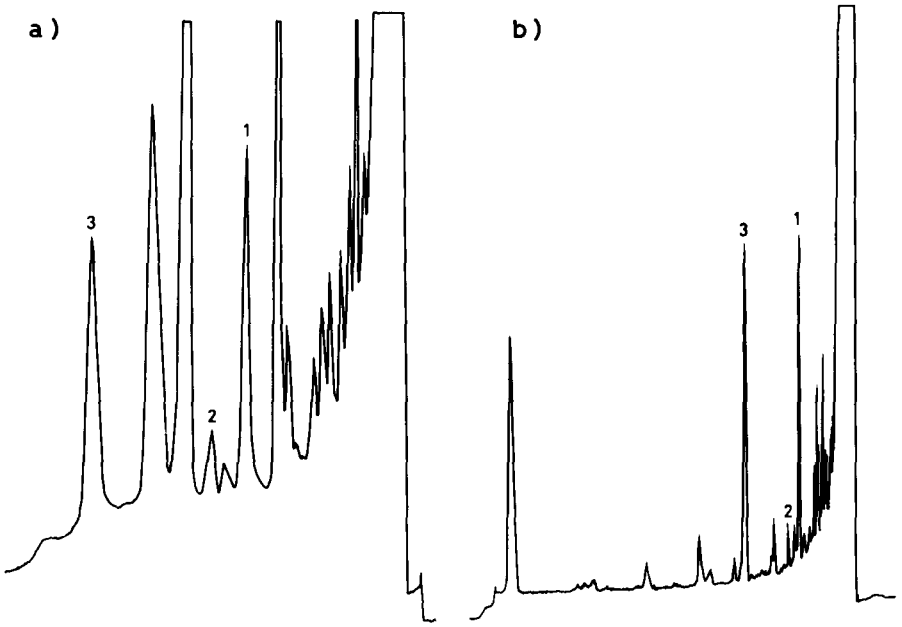

Fig. 1. Comparison of urine assays of methylhistidine isomers by packed (a) and capillary (b) chromatographic systems. In both analyses the amount injected corresponded to 1 μl of human urine. Peaks: 1 = 3-MH, 2 = 1-MH, 3 = internal standard.
Packed column GC conditions: a Fractovap 2150 gas chromatograph (Carlo Erba) equipped with a flame-ionization detector. The glass column (2 m × 4 mm I.D.) was packed with 1.5% SP-2250 and 1.95% SP-2401 on 100—120 mesh Supelcoport. Temperatures: oven 210°C, detector and injector 275°C. Carrier gas: nitrogen, flow-rate 33 ml/min.
Glass capillary GC conditions: gas chromatograph 3900-B (DANI, Monza, Italy) equipped with a flame-ionization detector. Capillary column (20 m × 0.85 mm O.D., 0.30 mm I.D.; Duran 50 with a 0.15 μm thick Pluronic F-68 coat). The split injection mode was used (flow 15 ml/min). Temperatures: oven 200°C, detector 280°C, injector 300°C. Carrier gas: hydrogen, flow-rate 0.7 ml/min.

TABLE I

PRECISION OF 1-MH AND 3-MH ASSAY

	Concentration (μg/ml)	*n*	C.V. (%)	
			1-MH	3-MH
Within-day	5.0	10	0.6	0.5
	50.0	10	0.7	0.5
Between-day	5.0	10	0.7	0.6
	50.0	10	0.8	0.7

TABLE II

COMPARISON OF 1- AND 3-MH LEVELS IN HUMAN URINE AND RAT MUSCLE ASSAYED BY GLASS CAPILLARY GC AND BY PACKED COLUMN GC

Sample	Creatinine excretion (mmol per 24 h)*	Methylhistidine level (μmol per 24 h)			
		Glass capillary GC		Packed column GC	
		1-MH	3-MH	1-MH	3-MH
Human urine*					
P.A.	7.31	58.30	184.80	50.00	185.20
M.R.	5.34	41.90	151.10	42.10	152.00
P.M.	3.50	61.08	112.29	61.94	112.83
S.A.	5.82	80.40	189.17	80.65	190.00
Mean ± S.E.	5.49 ± 0.95	60.33 ± 9.62	159.34 ± 19.72	60.92 ± 9.63	160.00 ± 19.29
	Protein in wet muscle (mg/g)				
Rat muscles**					
Palmaris longus	74.00 ± 2	1429 ± 7	888 ± 8	1430 ± 8	889 ± 7
Pectoralis	87.04 ± 2	2760 ± 5	664 ± 7	2759 ± 7	663 ± 5
Gastrocnemius	99.12 ± 3	1733 ± 8	774 ± 8	1730 ± 8	775 ± 4
Heart	105.12 ± 4	205 ± 4	196 ± 6	206 ± 3	197 ± 6

*Healthy youths (18—20 years), weight 60—70 kg, fed a diet containing no meat.
**Male CD-COBS rats (Charles River, Calco, Italy), body weight 250 g, eating a diet containing no meat. Muscles from two animals were pooled for each determination. Values are expressed as mean ± S.E.M. (n = 4).

The efficiency of the method (recovery) was tested for 1- and 3-MH derivatives from four spiked human urines and the same urines unspiked. Average recovery was 97 ± 2%. No significant differences were found between recoveries for different concentrations of either of the MH isomers (10, 25, 50, 75 ng). The reproducibility of the present method, summarized in Table I, was fairly good compared with that of GC using the glass capillary column [10]. Before developing the glass capillary GC method for 1- and 3-MH derivatives, we tried unsuccessfully to work with packed columns (SE-30, OV-1, OV-17, OV-101, 2% OV-17 + 1% OV-210), but there was no resolution between 1- and 3-MH peaks. The mixed stationary phase 1.5% SP-2250 and 1.95% SP-2401 on 100—120 mesh Supelcoport, however, shows excellent resolution properties as regards the two MH isomers.

Table II reports assays of 1- and 3-MH in human urine and rat muscles and compares the results with those obtained by the glass capillary GC method. The results agree well, confirming the reliability of the proposed method, associated with its greater simplicity, and suggest possible biomedical application in studies on muscle-wasting diseases. The stationary phase used has a long life and is therefore economical for routine use.

In conclusion, the newly developed GC method is satisfactory with respect to sensitivity, accuracy, precision and economy. The assay procedure is simple and convenient, and therefore clinically applicable to the routine analysis of 1- and 3-MH in biological samples.

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