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Note

Gas chromatographic separation of 3-methylhistidine

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The daily urinary excretion of the amino acid 3-methylhistidine (N^{τ}-methylhistidine, 3-Mehis) has been suggested as an *in vivo* index of muscle protein catabolism, because methylation of the histidine (His) residues occurs after their incorporation into the myofibrillar peptide chains, and the 3-Mehis released during protein catabolism is neither re-utilized nor metabolized, but is excreted quantitatively in the urine[1].

3-Mehis has been determined by ion-exchange chromatography with amino acid analyzers[2], but other methods of separation and determination include thin-layer chromatography[3–6], paper chromatography[3,7], paper electrophoresis[7,8] and spectrophotometry[9].

Gas–liquid chromatography (GLC) has been used for the separation and quantification of amino acids in biological fluids[10,11]. Gehrke and Leimer[10] have obtained good separation of and sensitivity to different natural amino acids by GLC of the trimethylsilyl (TMS) derivatives on a stationary phase of 10% of OV-11 on Supelcoport (100–120 mesh). This technique, however, has been criticized by Sarkar and Malhotra[11] on the basis of the multiple peaks found for several amino acids and complete absence of a His peak.

We have found no information in the literature on the separation of 3-Mehis by GLC, nor on the separation of 1-Mehis, another methylated histidine derived from anserine, also present in muscle. In our work with amino acids, we have used with success Gehrke and Leimer's method for the separation of 3-Mehis, 1-Mehis and His. This note, therefore, reports the GLC separation of the methylated histidines from histidine and other amino acids present in biological samples.

EXPERIMENTAL

An F & M model 402 high-efficiency gas chromatograph (Hewlett-Packard) with programmed temperature and dual flame ionization detectors was used in this investigation. The carrier gas was helium at a flow-rate of 45–75 ml/min. Two U-shaped glass columns (2 m × 2 mm) packed by vibration with 10% of OV-11 on Supelcoport (100–120 mesh) were conditioned according to Gehrke and Leimer[10].

The TMS derivatives of amino acids were prepared with bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and acetonitrile as indicated by Gehrke and Leimer[10].

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The amino acids derivatized for the study included 3-Mehis, 1-Mehis, His and a mixture of nine amino acids containing arginine (Arg), aspartic acid (Asp), cysteine (Cys), cystine (Cys-Cys), lysine (Lys), ornithine (Orn), phenylalanine (Phe), proline (Pro) and tyrosine (Tyr). Aliquots of aqueous solutions of amino acids were dried and derivatized for chromatography. Removal of water was carefully done with methylene chloride.

The experiments to be reported were designed to answer the following questions: (1) Can 3-Mehis be separated from 1-Mehis and His by GLC?; (2) is the response of 3-Mehis linearly related to concentration?; and (3) how would the 3-Mehis appear in a mixture of amino acids?

The first experiment was conducted isothermally at 210°C. This temperature was selected because of the elution temperature reported for histidine by Gehrke and Leimer[10]. The injections of the derivatives were 4 to 5 μl containing μg amounts of 1-Mehis, His and 3-Mehis (these concentrations of derivatives were used to ensure good peaks).

As shown in Fig. 1a–c, the TMS derivatives of His, 1-Mehis and 3-Mehis showed different retention times. Under the conditions of the experiment, 210°C isothermally, these retention times were 10.3, 8.8 and 7.5 min, respectively. A mixture of equal amounts of the methylated histidines showed a good separation (Fig. 1d).

In the second experiment, different amounts of the TMS derivative of 3-Mehis ranging from 5 to 50 ng were chromatographed isothermally at 210°C; Fig. 2 shows the linear relationship obtained when peak heights were plotted against 3-Mehis quantity.

The third experiment studied the elution of the TMS-methylated histidines

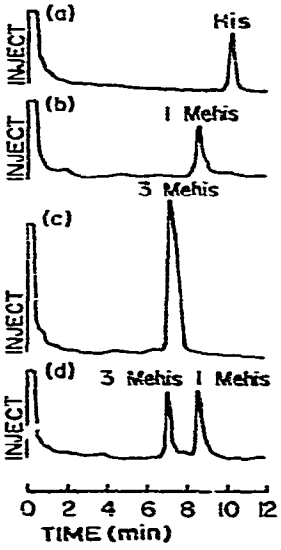

Fig. 1. Chromatographic separation of TMS derivatives of methylated histidines and His under isothermal conditions; column 210°C; detector 240°C; injector 250°C. Carrier gas flow-rate 45 ml/min.

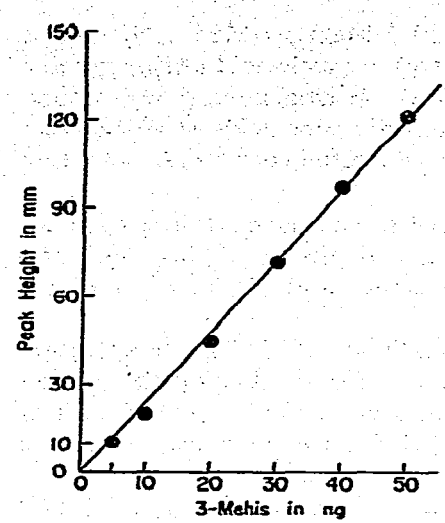

Fig. 2. Calibration curve for 5 to 50 ng of TMS derivative of 3-Mehis. Conditions as for Fig. 1, except carrier gas flow-rate was 75 ml/min.

and a mixture of amino acids using temperature programming. The starting temperature was 150°C (held for 3 min), and this was then programmed (at 4°C/min) to 240°C. At this time, the rate of temperature increase was changed to 10°C/min until the oven temperature was 270°C. Retention times and temperatures were calculated and are presented in Table I. With the programmed temperature, a good separation of amino acids was obtained, with the exception of Asp and Orn, which appeared as a single peak. The order of elution of amino acids is shown in Table I; for the methylated histidines and His it was 3-Mehis, 1-Mehis and His, the same as in the isothermal experiment. Under the temperature-programmed conditions described, 3-Mehis was separated from Tyr by 0.8 min and 3.3°C retention temperature, and from 1-Mehis by 0.5 min and 2°C. The differences in retention times and temperatures between Tyr and 3-Mehis, and between 3-Mehis and 1-Mehis, are sufficient to produce peaks of 3-Mehis adequate for GLC quantification.

TABLE I

RETENTION TIMES AND TEMPERATURES OF THE TRIMETHYLSILYL DERIVATIVES OF AMINO ACIDS

Amino acid	*Retention time (min)*	*Retention temp. (°C)*
Pro	3.2	150.7
Asp + Orn	8.7	172.7
Phe	9.5	176.0
Arg	10.7	180.7
Cys	12.3	191.3
Lys	14.0	194.0
Tyr	20.2	218.7
3-Mehis	21.0	222.0
1-Mehis	21.5	224.0
His	22.0	226.0
Cys-Cys	27.0	255.0

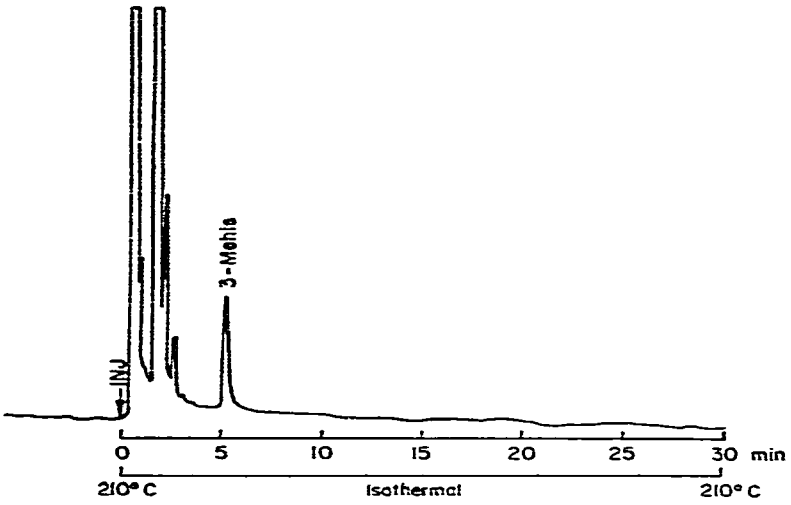

Fig. 3. Chromatogram of TMS derivative of 3-Mehis in urine cleaned-up by a modification of the Nishizawa *et al.* method of isolation of 3-Mehis[12]. Conditions as for Fig. 2.

COMMENTS

The purpose of the present report was to indicate the separation of the TMS derivatives of 3-Mehis and 1-Mehis from His and other natural amino acids using Gehrke and Leimer's technique[10]. It was shown that retention times under isothermal considerations were different for the methylated histidines and His, indicating a good separation of these amino acids. The response of different concentrations of the TMS derivative of 3-Mehis was shown to be linear, supporting the use of this technique for quantitative purposes.

The development of a GLC method to determine 3-Mehis in biological samples

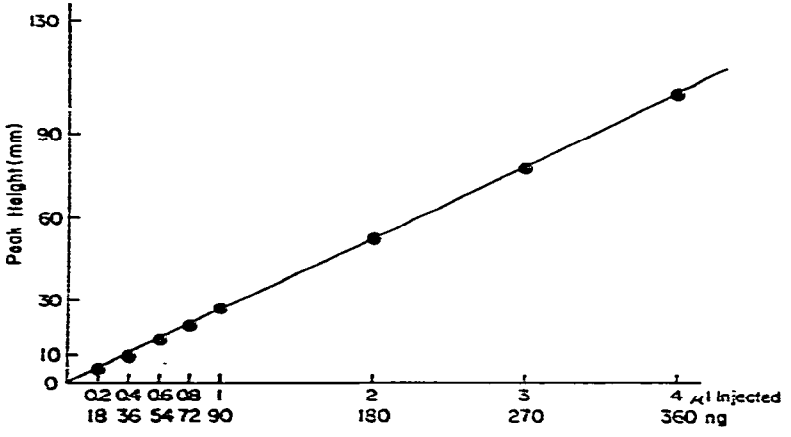

Fig. 4. Calibration curve using different injection volumes of urine prepared as indicated in Fig. 3.

would extend research in muscle protein catabolism *in vivo*, as GLC equipment is more readily available in most laboratories than amino acid analyzers or apparatus for HPLC. Samples of urine, the body fluid most desirable for analysis, present the complication of a clean-up step before derivatization. For this purpose, we have used a modified version of the procedure of Nishizawa *et al.*[12], and have obtained excellent isolation of 3-Mehis, as shown in Fig. 3. Further, a linear response was obtained (see Fig. 4) with urine covering a range from 18 to 360 ng, using a sample previously analyzed by other methods.

The present note supports Gehrke and Leimer's findings for His derivatization[10]. The problem referred by Sarkar and Malhotra[11] in the use of BSTFA for His derivatization is probably related to failure to eliminate water completely from the derivatization mixture. Differences in the amounts of amino acid used by these authors is an improbable explanation.

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* *Editor's Note:* See also F.-Y. Lieu and W. Jennings, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 3 (1980) 89.