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# GAS CHROMATOGRAPHIC DETERMINATION OF 1- AND 3-METHYLHISTIDINE IN BIOLOGICAL FLUIDS

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## SUMMARY

A gas chromatographic method for the determination of 1- and 3-methylhistidine in biological fluids was developed. The amino acid fraction containing 1- and 3-methylhistidine was isolated using ion-exchange chromatography. The amino acids were derivatized to N-trifluoroacetyl-O-isobutyl esters and analysed by gas chromatography with micropacked or capillary columns and an ionization-resonance detector. The conditions for the acylation of the histidines were studied and analyses of various derivatives such as N-trifluoroacetates, N-pentafluoropropionates and N-heptafluorobutyrates were tested. The detection limit of 1- and 3-methylhistidine was 50 ng per sample.

## INTRODUCTION

The amino acid 3-methylhistidine (3-MeHis) is a peptide-bound subunit of the contractile proteins actin and myosin. This amino acid is not re-used in the organism after protein degradation. It has been demonstrated [1–4] that 3-MeHis in biological fluids might be used as a marker of the rate of breakdown of myofibrillar proteins. Hence a highly sensitive and reliable method for the determination of this amino acid in biological samples is of practical importance. Little is known of the origin of the structural isomer of 3-MeHis, 1-methylhistidine (1-MeHis), and its role in muscle metabolism is still obscure. Therefore, the assay of 1-MeHis is likewise of interest.

Most of the known methods for the determination of 3-MeHis are based on various chromatographic analyses. Ion-exchange chromatography using traditional amino acid analysers [1,5] for the determination of 3-MeHis in urine has been reported. High-performance liquid chromatographic (HPLC) methods are particularly interesting because they make it possible to determine minimal amounts of amino acids, including 3-MeHis [6–8]. Gas chromatographic (GC) methods for amino acid analysis are highly attractive because of their simplicity

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and availability. Various GC procedures for 3-MeHis have been published [9–14] but, except for one [9], in which GC–mass spectrometry was used, they can be applied only to the analysis of urine samples with high 3-MeHis concentrations.

Our main purpose in this work was to develop a highly sensitive GC method for the determination of 1- and 3-MeHis.

## EXPERIMENTAL

### *Reagents and chemicals*

3-MeHis was purchased from Sigma (St. Louis, MO, U.S.A.), 1-MeHis from Koch-Light (Haverhill, U.K.), trifluoroacetic (TFAA), pentafluoropropionic (PFPA) and heptafluorobutyric (HFBA) anhydrides from Merck (Darmstadt, F.R.G.) and kits of amino acids from Reanal (Budapest, Hungary). All reagents and solvents were carefully dried and distilled.

### *Solutions*

Amino acids were dissolved in 0.1 *M* hydrochloric acid. The concentration of each amino acid was 2–3 μmol/ml. Both 1- and 3-MeHis were dissolved in 0.1 *M* hydrochloric acid at a concentration of 38 nmol/ml. Internal standards (metaphos and lindane for the capillary column) were dissolved in ethyl acetate at a concentration of 0.1 μg/ml. All solutions were cooled to 4°C.

### *Preparation of biological samples*

A 0.3-ml volume of 8% perchloric acid was added to 0.1 ml of serum. This mixture was kept at 4°C for 5 min, then centrifuged for 5 min at 600 *g*. The supernatant was applied to a 20×3 mm I.D. column filled with the ion-exchange resin KRS-8p ($H^+$) (0.1–0.25 mm) (Soiuzchim-reactive, Riga, U.S.S.R.). The column was washed with 2 ml of water and 3 ml of 1 *M* hydrochloric acid. The basic amino acids were eluted with 1 ml of 4 *M* hydrochloric acid. The eluate was evaporated under vacuum and the dry amino acid residue was transferred to a micro-reaction vessel (1 ml) (Supelco, Bellefonte, PA, U.S.A.) with two portions of 150 μl of methanol. The solvent was evaporated under vacuum and the residue was prepared for GC analysis using the procedure described below. To obtain the calibration graphs, aliquots of standard solutions of the amino acid mixture and 1- and 3-MeHis were applied to the ion-exchange column. When 1- and 3-MeHis were assayed in urine, 20 μl of urine were applied to the ion-exchange column.

### *Sample preparation for GC analysis*

The amino acid derivatives for GC analysis were prepared by the original procedure developed in our laboratory. Methylene dichloride (0.2 ml) was added to the dry residue of amino acids, contained in the micro-reaction vessel, and evaporated to dryness under vacuum. A 3 *M* solution of dry acetyl chloride in isobutanol was chosen as the esterification agent (the mixture was prepared directly before analysis from 720 μl of isobutanol and 280 μl of acetyl chloride); 0.2 ml of this esterification mixture was added to the residue. Each reactor was capped, mixed and left to react for 30 min at 120°C in a Multiblock heater. The samples

were evaporated to dryness under vacuum and the residues dried azeotropically with 0.1 ml of methylene dichloride. For N-acylation, 100 $\mu$l of tetrahydrofuran (THF) and 50 $\mu$l of TFAA were added and the vessels were than capped and heated in the Multiblock heater at 120°C for 15 min. The solvent and the reagent were removed under vacuum and the residue was dissolved in 20–50 $\mu$l of ethyl acetate. An aliquot of the internal standard solution was added before the GC analysis.

*GC conditions*

For GC analysis, an LHM-8 MD gas chromatograph (U.S.S.R.) equipped with a flame ionization detector and a Gasochrom-1109 gas chromatograph (U.S.S.R.) equipped with an ion-resonance detector were used. The operating conditions were as follows. The glass chromatographic column (1.5 m×1.2 mm I.D.) was packed with 3% OV-17 on 80–100 mesh Gas Chrom Q (Applied Science Labs., State College, PA, U.S.A.); the carrier gas was nitrogen at a flow-rate of 20 ml/min and the flow-rates of hydrogen and air to the detector were 30 and 300 ml/min, respectively; the injection port temperature was maintained at 250°C and the temperature of the column was programmed from 130 to 250°C at 20°C/min.

With the Gasochrom-1109 gas chromatograph, a glass column (2 m×1 mm I.D.) packed with 3% OV-17 on 100–120 mesh Chromosorb W HP (Hewlett-Packard, Avondale, PA, U.S.A.) was used. The sorbent was preliminary heated under vacuum at 350°C for 2 h. The carrier gas was nitrogen at a flow-rate of 12 ml/min; the auxiliary gas to the detector was nitrogen at a flow-rate of 65 ml/min. The injection port, detector and column temperatures were maintained at 260, 280 and 220°C, respectively.

A fused-silica capillary column (30 m×0.2 mm I.D.) with a methylsilicone liquid phase (Hewlett-Packard) was used in some experiments. The split injection mode was used. The carrier gas was hydrogen at a flow-rate of 1 ml/min and the splitter flow-rate was 30 ml/min. The auxiliary gas to the detector was nitrogen at a flow-rate of 70 ml/min. The column temperature was maintained at 205°C.

## RESULTS AND DISCUSSION

There are several critical points in the development of a GC method for 1- and 3-MeHis analysis: (a) the preparation of suitable derivatives of the amino acids; (b) the selection of a highly sensitive detection method; (c) the selection of an efficient column; and (d) the isolation of 1- and 3-MeHis from biological samples.

Most of the methods for the GC determination of amino acids are based on their conversion into N-perfluoro derivatives of O-alkyl esters [15,16]. We have previously determined amino acids as N-trifluoroacetates of O-*n*-propyl esters [17]. In some reports similar derivatives of 1- and 3-MeHis [10,12] were used in GC and it was shown that both the alkylation and acylation steps were quantitative.

Taking into account these data, we chose a similar two-stage procedure for GC analysis involving the N-trifluoroacetates of O-isobutyl esters of 1- and 3-MeHis. The analysis of standard mixtures showed that, in contrast to other workers [10], the yields of the alkylation and acylation reactions for 1- and 3-MeHis were con-

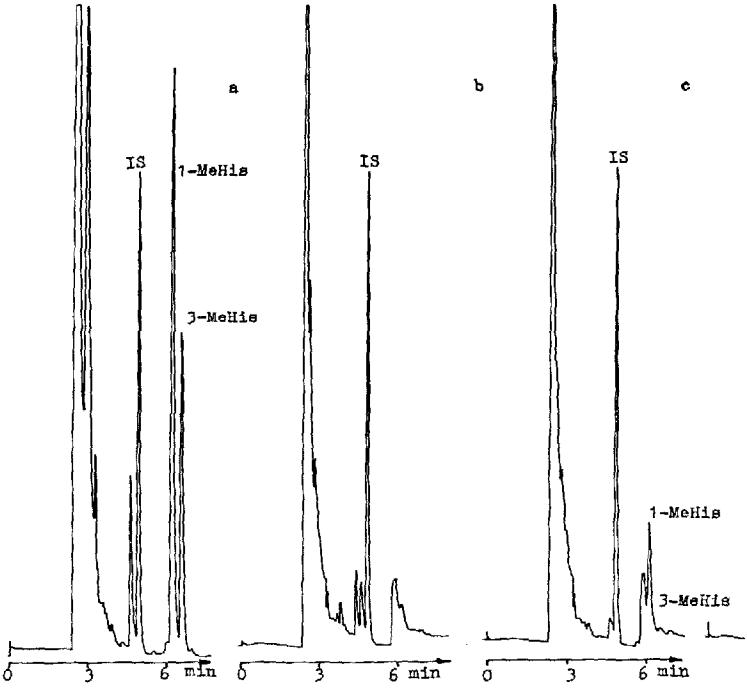
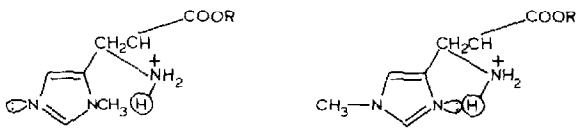



Fig. 1. Chromatograms of a mixture of 1- and 3-MeHis (3.1 nmol of each) *acylated with* 50 $\mu$l of TFA in 100 $\mu$l of (a) THF, (b) methylene dichloride and (c) ethyl acetate.

siderably different. Moreover, it was found that the results of the analysis did not depend on the alkylation conditions, but at the same time the acylation conditions (solvent, catalyst, etc.) influenced the 1- and 3-MeHis peak correlation (Fig. 1). The yields of 1-MeHis derivatives were almost always better than those of 3-MeHis derivatives. It was shown that 1- and 3-MeHis peaks become commensurable only in the presence of strong bases, such as pyridine. The difference in the reactivities of these isomeric compounds may be explained by the existence of an intramolecular hydrogen bond between the protons of the $\alpha$-amino group and the $sp^2$-hybridized nitrogen atom of the imidazole ring in 3-MeHis:




1-MeHis 3-MeHis

According to the results obtained, we considered that it was worthwhile acylating the methylhistidines in the presence of pyridine. However, more detailed investigations of pyridine as a catalyst for the acylation reaction showed that an increase in its amount usually led to the formation of ditrifluoroacetates, which were rapidly hydrolysed to monotrifluoroacetates. Moreover, the reaction course in the presence of pyridine was strongly influenced by the purity of the pyridine, moisture and other factors. A serious drawback with pyridine is its ability to form

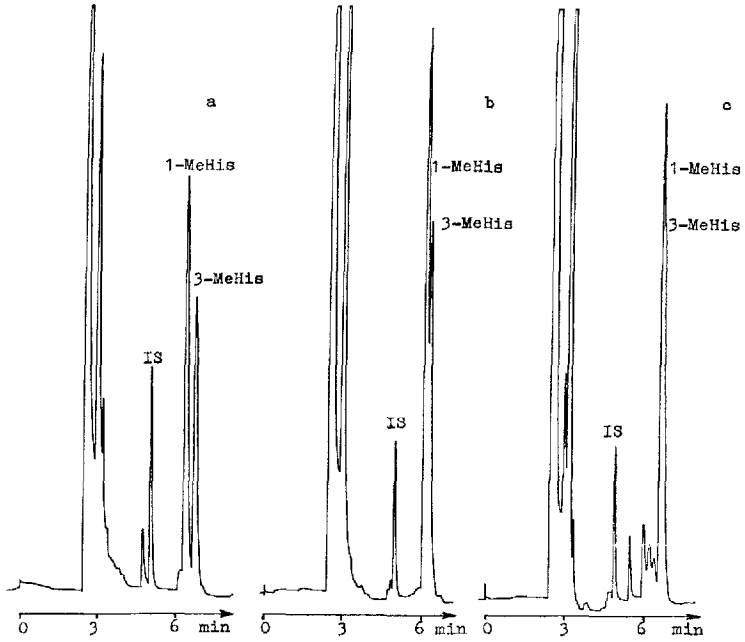



Fig. 2. Chromatograms of a mixture of 1- and 3-MeHis (3.1 nmol of each) acylated with 100 $\mu$l of THF by (a) TFA, (b) PFPA and (c) HFBA.

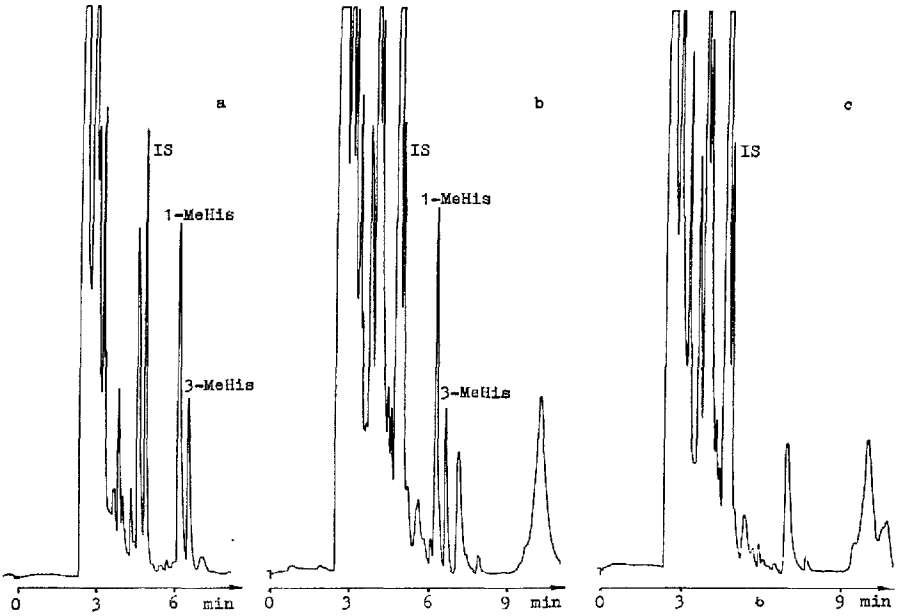



Fig. 3. Chromatograms of 4 *M* hydrochloric acid fractions after ion-exchange chromatography: (a) 1- and 3-MeHis (3.1 nmol of each); (b) 1- and 3-MeHis in a mixture with amino acids (2-3 $\mu$mol of each amino acid); (c) mixture of amino acids without 1- and 3-MeHis.

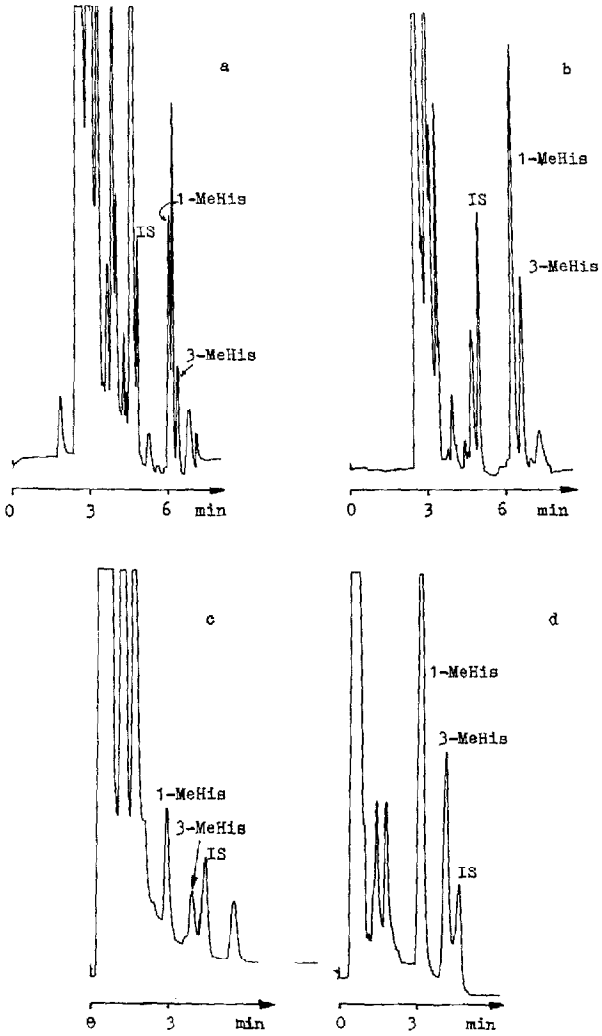



Fig. 4. Chromatograms of 1- and 3-MeHis isolated from biological samples on a capillary column, (a) serum (100 $\mu$l) and (b) urine (20 $\mu$l), and a micropacked column, (c) serum (100 $\mu$l) and (d) urine (20 $\mu$l).

TABLE I

CONCENTRATIONS OF 1-MeHis AND 3-MeHis IN HUMAN SERUM AND URINE

| Method | Concentration of 3-MeHis | | Concentration of 1-MeHis | |
|---|---|---|---|---|
| | Serum (nmol/ml) | Urine ($\mu$mol per 24 h) | Serum (nmol/ml) | Urine ($\mu$mol per 24 h) |
| This work | 8.8 ± 1.2 | 250 ± 52 | 8.0 ± 1.0 | 290 ± 90 |
| Mussini et al. [12] | | 159 ± 20 | | 795 ± 99 |
| Dohm et al. [18] | 7.6 ± 1.9 | 286 ± 51 | | |

pyridinium trifluoroacetate, which obscures the 1- and 3-MeHis, especially when micro- and nanogram amounts of these compounds are to be determined.

For this reason, in subsequent work THF was used both as a solvent and as a catalyst of the acylation reaction. It was shown that the corresponding 1-MeHis derivatives were recovered quantitatively, but the yield of 3-MeHis derivatives was only about 60%. This result may be explained by the lower basicity of THF compared with pyridine. Nevertheless, with THF, the reproducibility of the whole procedure was much better than with pyridine and the corresponding coefficients of variation were 2–3% at 1- and 3-MeHis concentrations of $1 \cdot 10^{-7}$–$1 \cdot 10^{-5}$ g ml$^{-1}$.

As mentioned above, one of the main problems was to select a highly sensitive method for the detection of 1- and 3-MeHis. The use of electron-capture detection (ECD) seemed to be the most promising as this method had been previously employed in amino acid analysis [15,16]. Comparing the two methods of detection, namely flame ionization (FID) and ion-resonance (IRD), which is analogous to ECD, it was established that the detection limit for 1- and 3-MeHis using FID was 150–200 pmol per injection and using IRD 0.3 pmol.

Satisfactory separations of 1- and 3-MeHis standards may be achieved using various stationary phases (PEGA, SP-2100, OV-17, etc.). However, complex amino acid mixtures (up to twenty protein amino acids) were best separated on capillary columns or with OV-17 micropacked columns.

In addition to the TFA derivatives of 1- and 3-MeHis O-isobutyl esters, we also studied the applicability of PFPA and HFBA in the acylation procedure. The chromatograms obtained using a capillary column and IRD are shown in Fig. 2, and indicate that the separation of the peaks of the 1- and 3-MeHis derivative deteriorates when HFBA is used instead of TFA. Taking into account these results and the fact that TFAA is less expensive than HFBA, TFA derivatives of 1- and 3-MeHis seemed more appropriate.

Traditional procedures for extracting amino acids from biological samples involve ion-exchange chromatography [13,14,17]. However, for the isolation of methylhistidines special methods are usually employed [12–14]. The main disadvantage of these methods is the necessity for large volumes of fluids (20–30 ml). We tried to develop a procedure for the isolation of methylhistidines that might be employed for biological samples of minimal volume containing minimal concentrations of amino acids. For this purpose, micro-columns with sulphocationite were used. Acidic and some neutral amino acids were eluted with 1 *M* hydrochloric acid, then 1- and 3-MeHis and basic amino acids were eluted with 4 *M* hydrochloric acid. The most basic amino acid, arginine, cannot be eluted from the column under these conditions. Thus the resin had to be washed with 4 *M* ammonia solution for regeneration. Fractions eluted from the column were analysed by GC. Chromatograms of a 1- and 3-MeHis standard fraction eluted with 4 *M* hydrochloric acid and the respective fractions of the amino acid mixture with and without 1- and 3-MeHis are shown in Fig. 3. In additional experiments it was established that the 1- and 3-MeHis losses did not exceed 5–7% when using our preparation procedure.

For the characterization of the proposed method and the determination of its application limits we have analysed different standards and biological samples.

The inter-analysis coefficient of variation for serum samples was 6–7% ($n=7$). The reproducibility was about 3% ($n=5$). Chromatograms for the determination of 1- and 3-MeHis in serum and urine on capillary and micropacked columns using IRD are shown in Fig. 4. The concentrations of 1- and 3-MeHis in biological fluids obtained using the described procedure and some literature data are listed in Table I. Our data agree with the results of other workers obtained using both GC [12] and HPLC [18] methods. Hence the proposed method can be used for the determination of 1- and 3-MeHis in 100 $\mu$l of serum and 2 $\mu$l of urine.

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