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## Note

# Determination of 3-methylhistidine in urine by high-performance liquid chromatography using pre-column derivatization with 9-fluorenylmethyl chloroformate

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3-Methylhistidine (3-MH) is formed by post-translational methylation of some histidine residues of the muscle proteins actin and myosin [1,2]. On degradation of these proteins, the 3-MH released is neither reutilized nor oxidized, but is quantitatively excreted in the urine [1]. It has been shown that urinary 3-MH originates mainly from skeletal muscle, with minor contributions from the skin and gastrointestinal tract [2]. Therefore, measurement of 3-MH in urine is considered a useful non-invasive technique to assess turnover of muscle protein and its degradation during several clinical conditions, e.g. malnutrition, sepsis and trauma [3].

Numerous analytical approaches to measure 3-MH in biological fluids have been used, including thin-layer chromatography [4], gas chromatography [5] and automatic amino acid analysis [6,7]. Several methods based on high-performance liquid chromatography (HPLC) have been described [8–17]. A sensitive detection of 3-MH can be attained by derivatization with reagents that yield strongly absorbing or fluorescent products. Derivatization can be performed in post-column mode, i.e. after separation by chromatography. Reagents used for this purpose include ninhydrin [6,7] and *o*-phthaldialdehyde [11,14,15,17]. Another approach is to perform derivatization in pre-column mode, i.e. before chromatography. The hydrophobic nature of the derivatized amino acids allows separation by reversed-phase chromatography. Two com-

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monly used reagents for pre-column derivatization are fluorescamine [9,10] and *o*-phthaldialdehyde [12,13], although both have their drawbacks. Fluorescamine requires prolonged incubation at elevated temperatures. The reaction with *o*-phthaldialdehyde proceeds rapidly at room temperature, but the products formed are not very stable. Therefore, reliable results can only be obtained by strict control of the interval between derivatization and chromatographic analysis, e.g. by automation of the entire procedure.

Recently, 9-fluorenylmethyl chloroformate has been shown to be an excellent reagent for the derivatization of both primary and secondary amino acids [18]. The reaction proceeds rapidly at room temperature, and the products formed are very stable. The aim of this study was to develop an analytical method for the determination of 3-MH in urine samples based on pre-column derivatization with 9-fluorenylmethyl chloroformate. Furthermore, the method should involve minimal sample handling and allow rapid isocratic separation by HPLC.

## EXPERIMENTAL

### *Reagents and chemicals*

3-Methylhistidine 1-methylhistidine, valine, DL-β-aminoisobutyric acid, sarcosine, lysine, cystine and asparagine were purchased from Sigma (St. Louis, MO, U.S.A.). Glycine, glutamic acid, aspartic acid, taurine, alanine, serine, proline, leucine, tryptophan, isoleucine, glutamine, arginine, phenylalanine, tyrosine, methionine, hydroxyproline and triethylamine were purchased from Merck (Darmstadt, F.R.G.). Threonine was obtained from Mann Labs. (New York, NY, U.S.A.), histidine from BDH (Poole, U.K.), cysteine from Koch-Light (Colnbrook, U.K.) and ethanolamine from J.T. Baker (Phillipsburg, NJ, U.S.A.). 9-Fluorenylmethyl chloroformate (FMOC-Cl) was supplied by Fluka (Buchs, Switzerland), N,N-dimethyloctylamine by Janssen Chimica (Beerse, Belgium) and acetonitrile by Bio-Labs. (Jerusalem, Israel). All other chemicals used were of analytical grade.

### *Instrumentation*

The HPLC equipment consisted of a Model 510 solvent-delivery system and a WISP Model 710B autosampler from Waters Assoc. (Milford, MA, U.S.A.). A Model SFM 23B fluorometer from Kontron (Zurich, Switzerland) with excitation monochromator set at 260 nm and emission monochromator set at 320 nm was used for fluorescence detection. Chromatographic separation was accomplished on a Spherisorb ODS column (200 mm × 3 mm I.D., 5 μm particle size) purchased from Chrompack (Middelburg, The Netherlands). The chromatograms were plotted and integrated using a Model C-R3A integrator from Shimadzu (Kyoto, Japan).

*Biological samples*

To determine reference values for excreted 3-MH, urine samples from seventeen male and sixteen female volunteers were analysed. After an overnight fast the first morning urine was discarded, and urine was then collected for the following 2 h. Samples were stored at −20°C before analysis. Urinary creatinine was measured by the kinetic Jaffé method.

*Derivatization and chromatography*

A 0.4-ml volume of urine or 3-MH standard was mixed with an equal volume of 1.0 *M* perchloric acid, and after 10 min the mixture was centrifuged at 1500 *g* for 10 min. An aliquot of the supernatant was diluted ten-fold with distilled water. A 0.1-ml aliquot of this dilution was mixed with 0.3 ml of distilled water, 0.1 ml of a 0.8 *M* borate buffer (pH 8.5), and 0.5 ml of a 15 m*M* solution of FMOC-Cl in acetonitrile. The reaction was allowed to proceed for at least 1 min at ambient temperature, and thereafter the excess of FMOC-Cl was removed by extraction with two 2.0-ml volumes of diethyl ether. The lower phase containing the derivatized amino acids was diluted five-fold with mobile phase before chromatography. The mobile phase was prepared by mixing equal volumes of acetonitrile and a 30 m*M* glycine buffer, adjusted to pH 3.0 with hydrochloric acid. The mobile phase was filtered before use through a 0.22-$\mu$m Durapor filter from Millipore (Milford, MA, U.S.A.). Isocratic elution was performed at a flow-rate of 0.7 ml/min.

## RESULTS AND DISCUSSION

Fig. 1A shows a chromatogram of derivatized 3-MH with a retention time of 13.2 min. The large peak with a retention time of 4 min is the hydrolysis product of the labelling reagent. The majority of the other amino acids tested eluted before or around the peak of the hydrolysis product and did therefore not interfere with the analysis. Lysine, histidine and tyrosine, which form doubly labelled derivatives, could not be detected. These hydrophobic derivatives were probably lost during the extraction with diethyl ether. In the procedure described by Einarsson et al. [18], extraction was carried out with pentane instead of diethyl ether to avoid loss of these derivatives. In our system, the selective removal of hydrophobic derivatives by extraction is advantageous, as it reduces the number of late-eluting compounds that might interfere with the detection of 3-MH. We found that repeated extractions with diethyl ether did not result in loss of 3-MH.

The only amino acid derivatives with retention times similar to 3-MH were found to be arginine and 1-methylhistidine. Fig. 1B shows a chromatogram of a set of amino acids including 3-MH, arginine and 1-methylhistidine. Arginine and 1-methylhistidine coeluted at 11.0 min and were well separated from the 3-MH peak. Attempts to reduce the peak width of 3-MH by addition of tri-

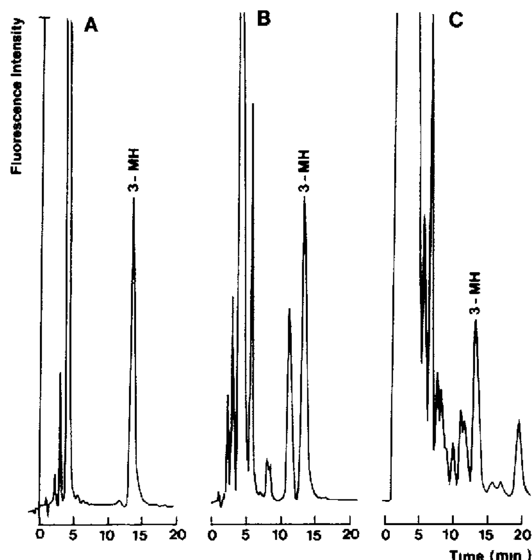


Fig. 1. (A) Chromatogram of standard 3-methylhistidine (100 nmol/ml) derivatized with 9-fluorenylmethyl chloroformate. (B) Chromatogram of a mixture of derivatized amino acids, including 1-methylhistidine, 3-methylhistidine and arginine. (C) Representative chromatogram of a derivatized urine sample. Conditions as in *Derivatization and chromatography*.

ethylamine to the mobile phase were unsuccessful. Peak width could be reduced by addition of 0.1% (v/v) of N,N-dimethyloctylamine to the mobile phase, but this resulted in a reduction of the retention time of 3-MH to such an extent that it was no longer separated from arginine and 1-methylhistidine.

A chromatogram of a derivatized urine sample is shown in Fig. 1C. In all urine samples the 3-MH peak showed baseline resolution. A peak eluting at ca. 19 min was present in some urine samples but did not interfere with the analysis if samples were injected every 15 min.

Several experiments to determine the overall performance of the 3-MH determination were done. A urine sample was analysed eight-fold in a single run, and the 3-MH level was calculated on the basis of peak-height or peak-area data. Using peak heights a coefficient of variation (C.V.) of 2.6% was obtained, whereas peak-area data obtained from the same chromatograms produced a C.V. of 4.0%. For this reason we routinely use peak-height data to calculate 3-MH levels.

Precision was further evaluated by analysing three urine samples on each of five consecutive days. The mean 3-MH concentrations obtained were 0.076, 0.149 and 0.224 m*M*, with C.V. values of 2.1, 1.7 and 5.4%, respectively. Analytical recovery of 3-MH added to six different urine samples was 104.6 ± 4.0% (mean ± S.D.). The detection limit at a signal-to-noise ratio of 3 was estimated to be 0.3 pmol of 3-MH. Calibration curves of peak height ($y$) versus amount

TABLE I

RESULTS OBTAINED FOR 3-METHYLHISTIDINE/CREATININE RATIOS IN URINE FROM HEALTHY ADULTS, IN COMPARISON WITH VALUES PUBLISHED BY OTHER INVESTIGATORS

| Ref. | Number of subjects | 3-MH/creatinine (mean ± S.D.) (mmol/mol) |
|---|---|---|
| Sjölin et al. [19] | 5 | 19.8 ± 1.3 |
| Mendez et al. [20] | 12 | 15.3 ± 1.9 |
| Elıa et al. [21] | 5 | 17.1 ± 2.5 |
| Long et al. [22] | 11 | 17 9 ± 1.1 |
| This study | 33 | 17.4 ± 4.6 |

of 3-MH injected ($x$) were linear over the range 1.0–100 pmol ($y = 50.14x - 0.77$) with a correlation coefficient of 0.9986.

Urine samples from 33 healthy volunteers were analysed. In order to reduce the contribution of exogenous 3-MH, which has an estimated half-life of ca. 12 h [19], the subjects fasted from 8.00 p.m. of the day before the day of urine collection. Furthermore, the first morning urine was discarded as it was expected to contain a relatively large amount of 3-MH from dietary origin. In all samples 3-MH could be measured without interference from other components. The 3-MH excretion was expressed as the molar ratio of 3-MH and creatinine. In this way it is not necessary to collect 24-h urine samples [19]. From Table I it can be seen that the values obtained by the present method are in close agreement with values reported by other investigators.

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