*Journal of Chromatography*, 182 (1980) 414—418
*Biomedical Applications*


CHROMBIO. 556

Note

# Rapid high-performance liquid chromatographic method for quantitation of 3-methylhistidine

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(First received October 16th, 1979; revised manuscript received December 13th, 1979)

The amino acid 3-methylhistidine is closely associated with skeletal muscle metabolism. The vast majority of the 3-methylhistidine formed in the body is present in skeletal muscle [1]. Methylation of the histidine occurs after its incorporation into the peptide chains of actin and myosin [2—4] and after catabolism of these proteins the liberated 3-methylhistidine is not recycled but quantitatively excreted in the urine of animals [5] and man [6]. The total amount of 3-methylhistidine excreted in the urine has therefore been proposed as an index of muscle protein catabolism [5].

Previous analyses for 3-methylhistidine in biological materials have predominately relied upon ion-exchange chromatography [7] and automatic amino acid analysis [8—10].

The importance of 3-methylhistidine as an index of muscle protein turnover shares considerable interest by many investigators, however, the time required for the analysis of 3-methylhistidine is relatively long and so it limits the application of this method for clinical studies. This report describes a simple, rapid high-performance liquid chromatographic (HPLC) technique which utilizes a reversed-phase separation with ion-pairing and post-column fluorescence derivatization for the analysis of 3-methylhistidine.

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## EXPERIMENTAL

### *Materials*

All reagents used were of highest purity available (A.C.S. certified grade). Boric acid, β-mercaptoethanol, potassium hydroxide (Fisher Scientific, Pittsburgh, PA, U.S.A.), *o*-phthalaldehyde (Eastman Kodak, Rochester, NY, U.S.A.), Brij 35, 30% solution (Pierce, Rockford, IL, U.S.A.), and ethanol (U.S. Industrial Chemicals, New York, NY, U.S.A.), were used in preparing the derivatization reagent. L-3-Methylhistidine, L-1-methylhistidine and L-histidine were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium hexanesulfonate was provided from the Peptide Synthesis Group (Massey University, Palmerston North, New Zealand).

### *Methods*

The *o*-phthalaldehyde reagent was prepared by dissolving 30 g of boric acid in 1.0 l of deionized water, adjusting the pH to 10.4 with potassium hydroxide pellets, filtering through a 0.45-μm aqueous filter (Millipore Bedford, MA, U.S.A.) and adding 1.0 ml of 30% (w/v) aqueous solution of Brij 35, then transferring the solution into a dark glass bottle. Separately, a solution containing 600 mg of *o*-phthalaldehyde and 200 μl of β-mercaptoethanol in 10 ml of ethanol was added to the borate solution and stored under nitrogen. The reagent was prepared daily.

A stock solution of 5 m*M* sodium hexanesulfonate, pH 3.2 (pH adjusted with glacial acetic acid) in deionized water was degassed prior to use as the mobile phase in the chromatograph.

A standard solution of 3-methylhistidine was prepared in deionized water and analyzed by HPLC. When not in use, the solution was stored at 4°C.

Urine was collected from three children and one adult for 24 h in toluene and aliquots were stored at −15°C until analysis. Following the addition of 10% solution of trichloroacetic acid to the urine (1:9, v/v), the sample was centrifuged, the supernatant adjusted to pH 3.5 with 10% trichloroacetic acid and aliquots were injected into the chromatograph for the separation and quantitation of 3-methylhistidine.

### *Apparatus*

A Model 6000A solvent delivery system and U6K universal injector (Waters Assoc., Milford, MA, U.S.A.) were used with the HPLC system. The fluorescence detector was a Model 420 using an excitation filter of 340 nm and an emission filter of 440 nm (Waters Assoc.). The HPLC column used was stainless steel (300 × 4 mm I.D.) μBondapak $C_{18}$, particle size 10 μm, with a guard column, 2 in. long and 0.5 ml volume capacity, packed with Bondapak Phenyl/Corasil, particle size 37—50 μm and a post-column mixing chamber, all from Waters Assoc.

The emission of 440 nm was monitored at a chart speed of 5 mm/min on a 10-mV linear recorder B-5000 OmniScribe (Houston Instrument, Austin, TX, U.S.A.). The peak heights were measured.

A schematic diagram of post-column fluorescence derivatization is given in Fig. 1.

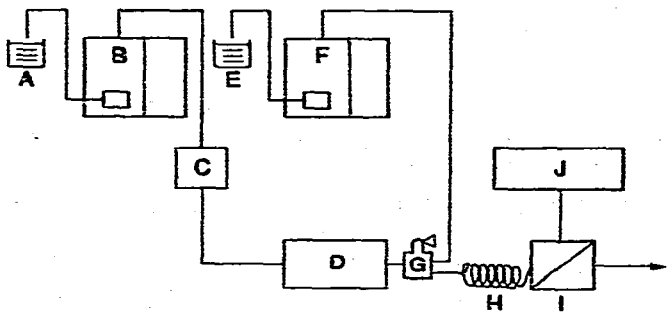


Fig. 1. Schematic diagram of post-column fluorescence derivatization system. A, reservoir (5 m*M* sodium hexanesulfonate); B, pump; C. injector; D, μBondapak $C_{18}$ column; E, reservoir (*o*-phthalaldehyde); F, pump; G, mixing chamber; H, stainless-steel loop; I, fluorescence detector; J, recorder.

## RESULTS AND DISCUSSION

The analysis of histidine and its metabolites in the presence of a large number of other materials present in urine, requires the addition of hydrophobic ion-pairing reagents to the mobile phase. At pH 3.2 hexanesulphonate significantly increases the retention of histidine, 1- and 3-methylhistidine. This increase is presumably due to ion-pairing of cationic groups formed at pH 3.2 from the α-amino- and imidazole N-groups in the sample molecules, with the hexanesulphonate in the mobile phase. Such an association has previously been shown to cause an increase in retention of amino acids on reversed-phase chromatography [11].

The use of sodium hexanesulphonate allows excellent resolution of histidine and the similar structural isomers 1- and 3-methylhistidine. This resolution is dependent, however, on the carbon-chain length of the sulphonate as the other members of the $C_4$—$C_8$ series did not give as good a separation, particularly in the urine samples.

3-Methylhistidine was identified on the basis of retention time by comparison with standards. As is shown in Fig. 2, good separation of 3-methylhistidine is obtained from other components present in human urine. For further confirmation of chromatographic peaks, work is in progress to utilize high-resolution mass spectrometry.

3-Methylhistidine added to urine was analyzed with good precision at concentrations comparable to those in samples of biological fluids. Repeated injections of 0.1—10.0 μg of 3-methylhistidine gave an overall coefficient of variation less than 5%. Recovery of 3-methylhistidine added to urine samples was at least 99%.

A linear response was observed for concentrations of 3-methylhistidine ranging from 100 ng to 10 μg injected into the column, thus, providing a more than adequate range for the analyses of 3-methylhistidine levels at concentrations found in biological materials (approximately 50—100 ng/μl urine).

The excellent sensitivity of this procedure is partly due to the fact that isocratic conditions can be used to separate histidine and its metabolites from the other urinary components. In addition, the analysis can be carried out

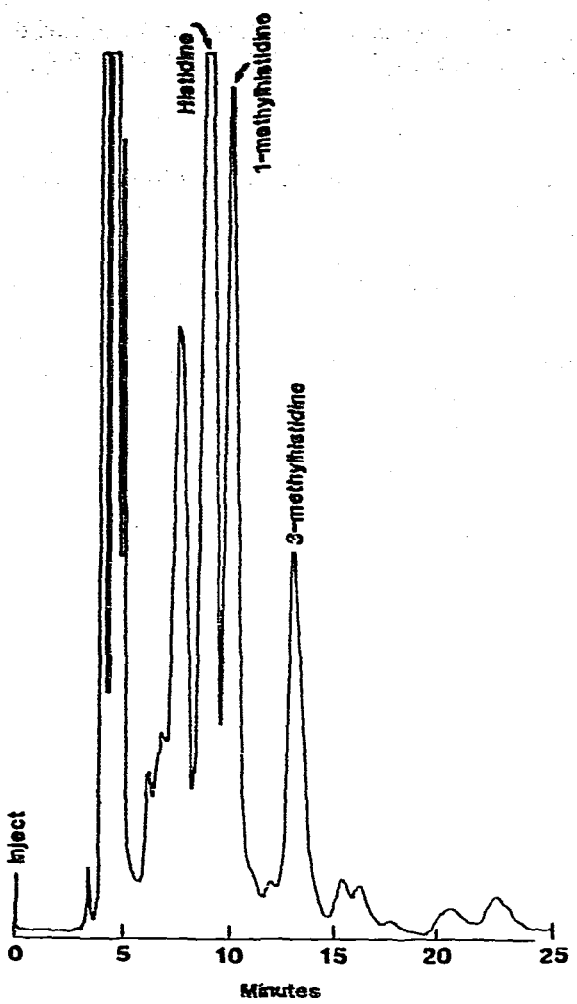


Fig. 2. Reversed-phase HPLC separation of 3-methylhistidine of urine. Sample injected, 5 $\mu$l; column, $\mu$Bondapak $C_{18}$ (300 × 4 mm I.D.) and mobile phase, 5 m*M* sodium hexanesulfonate at a flow-rate of 0.8 ml/min. Post-column derivatization occurs at the mixing chamber with *o*-phthalaldehyde (1 ml/min). Separation and quantification is via fluorescent detector Model 420 excitation 340 nm, emission 440 nm, and a linear chart recorder.

rapidly using the 30-min conditions shown in Fig. 2. Any non-polar contaminants which were retained on the column was washed with acetonitrile at the end of each day's analyses.

The reversed-phase partition mode of HPLC with post-column derivatization with *o*-phthalaldehyde and fluorescence detection for separation, provides a rapid (16-min baseline separation), highly sensitive, simple and quantitative method for the analysis of 3-methylhistidine at the nanogram level.

ACKNOWLEDGEMENTS

We are indebted to Dr. David Harding (Massey University) for synthesizing

and providing the hexanesulfonate and to Ms. Letitia B. Hendrix for secretarial expertise. This study is partially supported by Grant No. NICHD HD 13507-02.

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