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Note

Simple and rapid high-performance liquid chromatographic method for the quantification of 3-methylhistidine

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The amino acid 3-methylhistidine (3-MeHis) is a non-reutilised amino acid present in the myofibrillar proteins actin and myosin [1]. The amino acid is quantitatively excreted in the urine in proportion to the rate of degradation of the myofibrillar proteins [1], thus providing a non-invasive technique for measuring the rate of myofibrillar protein breakdown in man [2]. The low concentration of 3-MeHis in urine, 0.05—0.20 m*M*, particularly relative to that of other amino acids, has led to the development of specific analytical techniques [3]. These methods have made use primarily of conventional automatic amino acid analysers (see ref. 4): however, recently high-performance liquid chromatographic (HPLC) methods have become available [5—7].

The present report describes an HPLC method which is both simple and rapid yet sensitive and which does not require the use of fluorimetric detection [6, 7].

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EXPERIMENTAL

Equipment

A Model 6000A solvent delivery system with a U6K universal injector (Waters Assoc., Milford, MA, U.S.A.) was used in the present studies. Chromatographic separations were performed using a stainless-steel HPLC column (300 × 4 mm I.D.) packed with μBondapak C_{18}, particle size 10 μm (Waters Assoc.). A guard column (0.5 ml capacity) was used containing Bondapak Phenyl/Corasil, particle size 35—50 μm (Waters Assoc.).

The absorbance of the column eluent was monitored at 405 nm using a Model 440 absorbance detector (Waters Assoc.) fitted with a 405-nm filter and aperture plate. Absorbance was recorded on a linear chart recorder (Omniscribe B-5000; Houston Instruments, Austin, TX, U.S.A.) and quantified by measuring the peak heights.

Materials

All reagents were of the highest purity available. Amino acids and *o*-phthalaldehyde (OPT) were obtained from Sigma (St. Louis, MO, U.S.A.). Other reagents were purchased from BDH Chemicals (Poole, Great Britain) or from Waters Assoc.

Methods

Ninhydrin—*o*-phthalaldehyde (ninhydrin—OPT) reagent was prepared by adding OPT (5 g l^{-1}) to ninhydrin reagent prepared according to the description of Spackman et al. [8] using titanium trichloride as the reducing agent [9]. The reagent was stored under nitrogen in a dark bottle.

Urine samples were collected using glacial acetic acid as preservative and stored at —20°C to await analysis.

Samples were prepared by adding 0.5 ml of the ninhydrin—OPT reagent to 1 ml of filtered urine or a solution of 3-MeHis. The solution was mixed thoroughly and allowed to react at 40°C for 10 min in a water-bath. To this solution were added 4.0 ml of ethyl acetate; the solution was then mixed thoroughly and cooled in ice. The solutions were centrifuged at low speed to separate the aqueous and organic phases. Aliquots, normally 25 μl, of the aqueous phase containing 3-MeHis were injected directly into the chromatograph. The eluent was water—methanol (60:40), degassed with helium. The eluent flow-rate was 1 ml min^{-1}.

Results are expressed as mean ± standard error of the mean (number of determinations).

RESULTS AND DISCUSSION

Calibration curves for 3-MeHis prepared using standard 3-MeHis solutions were linear for the range up to 5.0 μmol ml^{-1} 3-MeHis assayed, thus providing an adequate range for the concentration of 3-MeHis in urine. The colour yield, determined spectrophotometrically, was $6.97 \cdot 10^3$ l mol^{-1} cm^{-1} at 405 nm for 1.0 *M* solution of 3-MeHis. Blank absorbance was 0.077 ± 0.003 (n=6) units. The precision of determination was good; replicate samples of 0.15 μmol

ml^{-1} 3-MeHis analyzed yielded a coefficient of variation of 7.0% (n=8).

A typical chromatogram obtained for the analysis of 3-MeHis is shown in Fig. 1. An excellent separation of 3-MeHis from other urinary components was obtained. Recovery of 3-MeHis added to urine was 100.4 ± 1.7% (n=8). Characterization of the 3-MeHis peak in urine samples was confirmed by co-chromatography with authentic 3-MeHis using different elution solvent mixtures. Using the present procedure the daily 3-MeHis excretion by healthy children, approximately 25 kg in body weight and receiving a meat-free diet, was 15.6 μmol 3-MeHis $mmol^{-1}$ creatinine 24 h^{-1}, a value similar to those obtained using ion-exchange chromatography (12.6 μmol $mmol^{-1}$ 24 h^{-1}) [2] and fluorescence HPLC (14.9 μmol $mmol^{-1}$ 24 h^{-1}) [7].

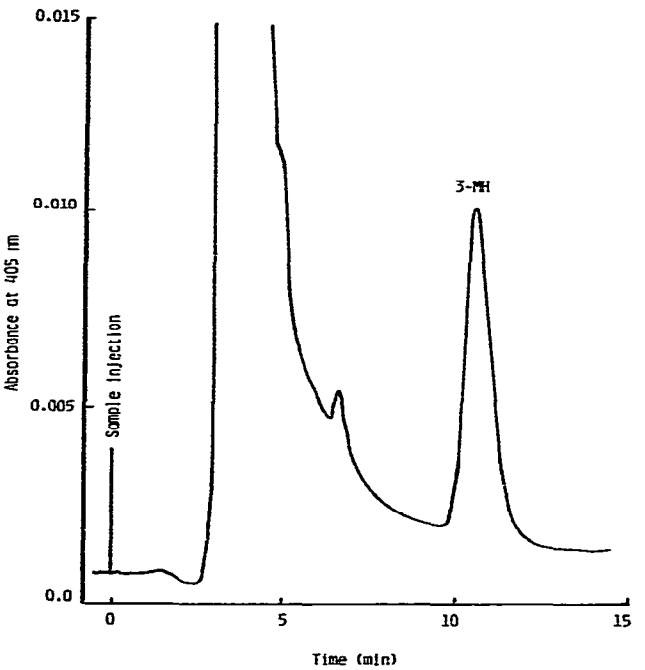

Fig. 1. Chromatographic separation of 3-methylhistidine (3-MH) in urine. The chromatogram was recorded using 1 ml of urine prepared as described in the text. 3-Methylhistidine represents 1.97 nmol. Other reaction products are rapidly eluted with the sample solvent front.

The analysis of 3-MeHis in the presence of many other components in urine requires special analytical methods [3]. The reaction mechanism between 3-MeHis and ninhydrin—OPT and the nature of the chromogenic product formed are not yet known. The reagent, previously shown to be semi-specific for 3-MeHis, is, however, an effective chromatographic reagent for the detection and quantification of 3-MeHis by ion-exchange chromatography [3]. The adaptation of this method to HPLC considerably simplifies and increases the sensitivity of the analytical technique when compared with conventional ion-exchange methods. The method is rapid and quantitative and requires only a basic model liquid chromatograph suitable for isocratic elution with spectrophotometric detection at 405 nm.

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