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Determination of urinary 3-methylhistidine by high-performance liquid chromatography*

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Introduction

The requirement of specific and inexpensive techniques for monitoring urinary 3-methylhistidine (3-MH) has stimulated method development using both traditional techniques, such as amino acid analysis [1–2] and colorimetry [3–5], and more recent techniques, such as GLC [6] and HPLC [7]. Unfortunately all these methods require peculiar technological requirements or time consuming sample workup procedures, incompatible with the needs of a clinical control laboratory.

For these reasons a new, inexpensive, yet accurate and precise analytical method, that is simple to use routinely for the characterization and determination of urinary 3-MH has been developed.

Reversed-phase HPLC was selected because of the complex nature of the urinary matrix. 3-MH reacts with *o*-phthaldehyde (OPA) to yield an isoindole which possess an absorbance maximum at 405 nm which enables operation in the visible region. Furthermore at this wavelength interference from several urinary catabolites is eliminated and a sensitivity in the nanogram range is achievable. The evaluation of the method is described in the present communication.

Experimental

Materials and equipment

Chemicals used in the investigation included, pure 3-methylhistidine (Sigma), ninhydrine/OPA reagent kit (Sigma), methanol and chloroform (Merck), acetic acid and

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ammonium acetate (C. Erba). Sample clean-up was achieved using 10 SPE C18 columns (Baker). Analyses were performed using a Hewlett–Packard liquid chromatograph HP 1090 M equipped with a UV-Vis diode array detector HP 1040 M and a workstation HP 9000 mod. 310.

Analytical conditions

Separations were performed using a 250 × 4 mm i.d. Supelcosil LC-1 column (5 μm spherical particles) linked in series with a 250 × 4 mm i.d. Lichrosorb RP-2 column (10 μm irregular particles). A 4 × 4 mm i.d. Lichrosorb RP-18 guard column was placed before the analytical column system. Elution was effected by gradient elution with mobile phases A = 0.01 M aqueous acetate buffer adjusted to pH 3.5 with acetic acid and B = methanol with the following elution profile: 40% B for 5 min, then a linear gradient to 80% B in 6 min; 80% B for 7 min, then a linear gradient back to 40% B in 7 min. A flow rate of 1.0 ml min^{-1} was used throughout and analytes detected by absorption measurements at 405 nm.

The chromogenic reagent (ninhydrin/OPA) was prepared by adding *o*-phthalaldehyde to ninhydrin, according to Spackman [8], using titanium trichloride as reducing agent.

Stored in the dark and under nitrogen, the reagent, keeps its activity for at least 10 days.

Sample preparation

1 ml of urine (or 3-MH solution) is added to 0.5 ml of ninhydrin/OPA reagent in a crimp top vial, shaken on a vortex shaker for 8 min, heated in a water bath at 40°C for 5 min and then chilled to 0°C. An aliquot (0.5 ml) of the reaction mixture is transferred to a sample clean-up column and filtered under pressure. The filtrate is added to 3 ml of chloroform, shaken for 3 min and centrifugated at 1500 r.p.m. for 10 min. A 10 μl volume of the organic layer is injected into the liquid chromatograph.

Results and Discussion

A typical chromatogram of 3-MH is shown in Fig. 1 which reveals a retention time of 13.5 min on the combined LC-1, RP-2 columns.

Quantitative assays were performed by means of the external standard procedure, using a calibration curve obtained from aqueous solutions of 3-MH in the range 0.03–0.6 μmol ml^{-1}. In this range the detector response was linear, the regression data being $y = 3.69x + 6.73$ ($n = 5$) with a correlation coefficient of 0.995. Thus establishing that the analytical conditions described enable the fast and reliable identification and determination of urinary 3-MH.

The coupling of the LC-1 and RP-2 columns, having a similar polarity, enables the separation of 3-MH from other urinary compounds, as demonstrated in Fig. 2. The identity of the 3-MH peak was confirmed by comparing the chromatograms acquired at three wavelengths (405, 355 and 410 nm) for the 3-MH peak in a standard solution and in biological sample (Figs 3a and b). The 3-MH peak purity in biological samples was evaluated by recording the normalized apex and slope spectra and comparing them with the same spectra for a reference sample of 3-MH. Several ratio chromatograms were generated as further confirmation of peak purity; the ratio between chromatograms at different wavelengths resulted in a straight line for the 3-MH peak both for the standard and for the sample, confirming the absence of coeluted compounds.

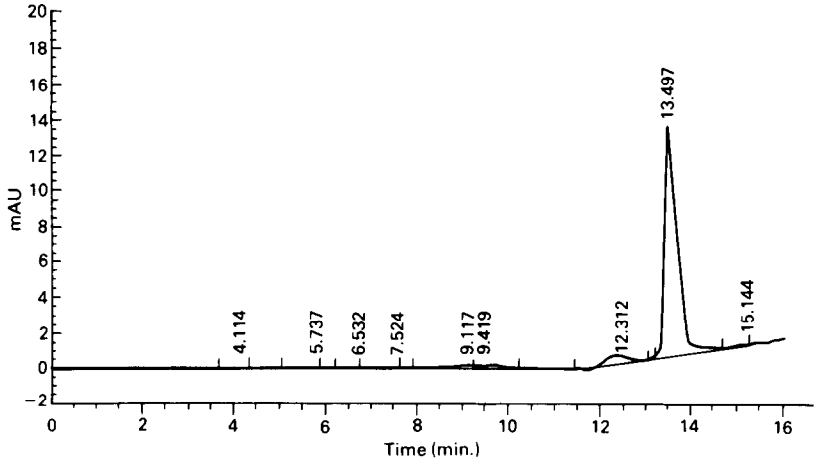

Figure 1
A typical chromatogram of standard solution of 3-methylhistidine obtained using the LC-1, RP-2 column combination with methanol–aqueous 0.01 M acetate buffer pH 3.5 gradient elution.

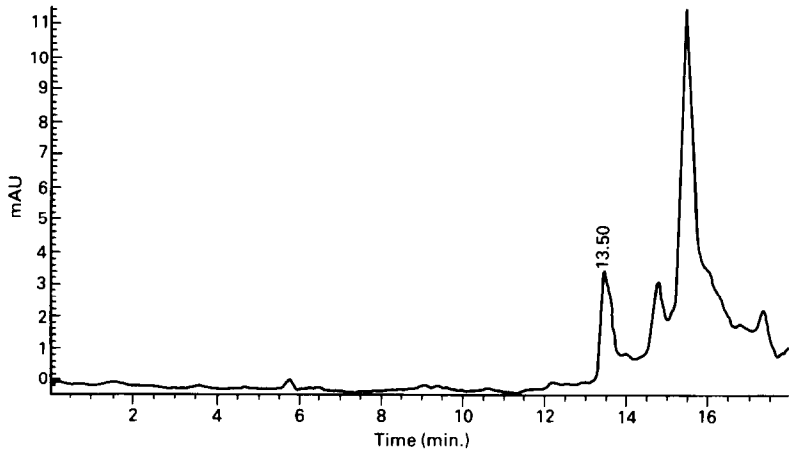

Figure 2
Chromatogram of human urine sample obtained using instrumental conditions as in Fig. 1.

The precision of the quantitative determination was tested by analyzing five different aliquots of the same urine sample. The 3-MH content was found to be 198 μmol l^{-1} ± 2.5 RSD(%). The accuracy of the method was evaluated analyzing five urine samples with known 3-MH content, the recovery was 98% ± 1.4 RSD(%).

Also the described method was used to determine the 3-MH content of urine samples taken from eleven children (age 4–11) who had been kept on a meat-free diet. A 3-MH average content of 0.158 μmol ml^{-1} was observed over a period of 24 h, value in good agreement with literature results [9–10].

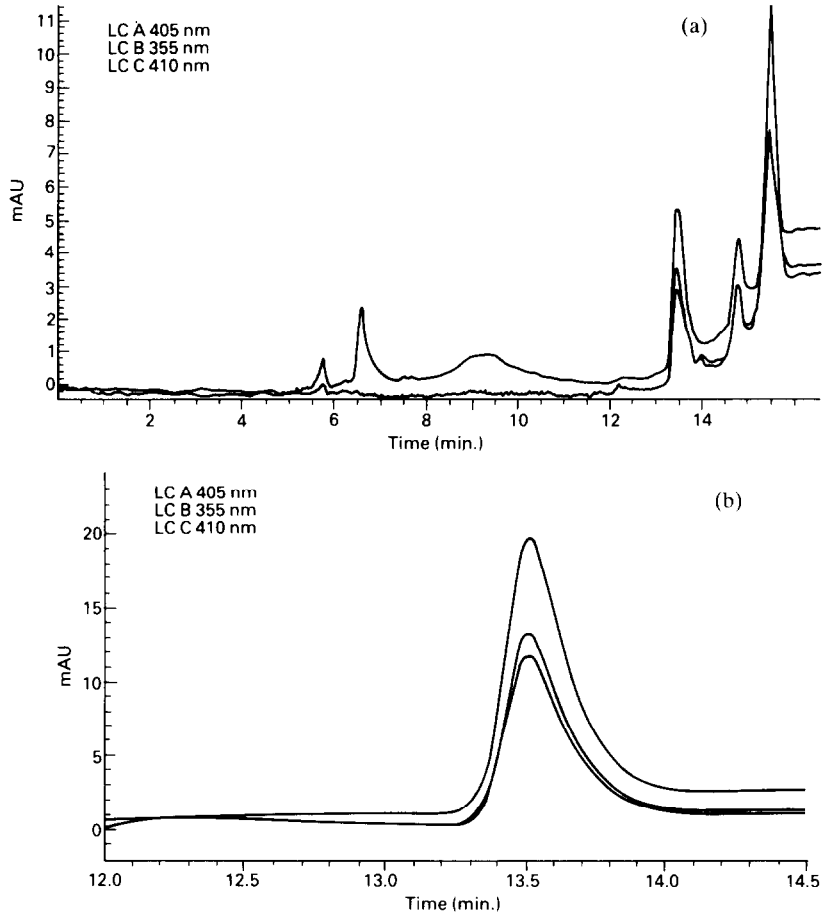

Figure 3 (a) Trace of amplified 3-MH standard signal detected at 405 nm, 355 nm, 410 nm. (b) Trace of the human urine sample detected at 405 nm, 355 nm, 410 nm.

Conclusion

A liquid chromatographic method has been developed for the determination of urinary 3-methylhistidine that is precise and inexpensive which satisfies the requirements for the clinical monitoring neuromuscular diseases (e.g. Duchênne muscular dystrophy).

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