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Short Communication

Determination of urinary 3-methylhistidine by high-performance liquid chromatography with *o*-phthaldialdehyde precolumn derivatization

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ABSTRACT

A high-performance liquid chromatographic method to measure the concentration of 3-methylhistidine in human urine with *o*-phthaldialdehyde precolumn derivatization is described. A clear separation of the 3-methylhistidine derivative from other derivatives in urine was achieved within 25 min. The linearity, recovery and coefficients of variation were determined. The method was succesfully applied to clinical assays and used for diagnostic purposes.

INTRODUCTION

3-Methylhistidine constitutes an integral component of both actin and myosin [1]. It is formed by post-translational modifications of certain histidine residues of the polypeptide chain [2]. After being released during protein degradation, 3-methylhistidine cannot be reutilized for protein synthesis in muscle, unlike other amino acids, as it lacks its specific tRNA [2]. Further, it is not metabolized to any substantial extent and is rapidly excreted in the urine [3]. As more than 90% of the total human 3-methylhistidine pool exists in skeletal muscle [4], its urinary excretion in humans has been used as a biological marker for skeletal muscle protein breakdown [5–10].

Traditional techniques for determining urinary 3-methylhistidine involved ion-exchange chromatography followed by postcolumn derivatization with ninhydrin or with a fluorogenic reagent [10–12]. These techniques have the disadvantages that they are laborious, the analysis times are long (over 1 h) and they require dedicated instrumentation.

This study was undertaken to develop a procedure for the routine therapeutic monitoring of the concentration of 3-methylhistidine in patients suffering from gastric cancer. A technique that has been used for the determination of 3-methylhistidine in urine is described. The technique is based on derivatization of the 3-methylhistidine and its separation from other derivatives in urine using high-performance liquid chromatography (HPLC) and either UV–visible or fluorimetric de-

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tection. The derivatization reaction is rapid and complete within 60 s and the products can be injected immediately. The derivatives are separated within 25 min.

EXPERIMENTAL

Chemicals

3-Methylhistidine standard was purchased from Sigma (St. Louis, MO, USA) and *o*-phthaldialdehyde from Fluka (Buchs, Switzerland). HPLC-grade methanol, analytical-reagent grade ethanolamine (internal standard) and other reagents were Chinese commercial products. High-purity water was obtained with an Elgastat UHP system (Elga, Elgale, UK).

Standard solution and derivatization reagent

A stock standard solution of 3-methylhistidine was prepared in 0.1 *M* hydrochloric acid at a concentration of 2.5 mmol/l and was kept in a refrigerator. A working standard solution of 3-methylhistidine (200 μmol/l) was prepared by dilution. An internal standard solution containing 400 μmol/l ethanolamine was prepared in 0.1 *M* hydrochloric acid.

The *o*-phthaldialdehyde reagent was prepared by dissolving 50 mg of *o*-phthaldialdehyde in 4.5 ml of methanol and diluting to 5 ml with 50 μl of sulphur alcohol and 0.4 *M* sodium borate buffer (pH 11.2). The reagent mixture was kept in the dark at 4°C. Fresh *o*-phthaldialdehyde reagent was prepared every two weeks.

Apparatus

A Hewlett-Packard (Palo Alto, CA, USA) Model 1090 M liquid chromatograph equipped with a Hewlett-Packard Model 1046 A programmable fluorescence detector, a Hewlett-Packard diode-array UV detector and a Beckman Microfuge E were used.

Chromatographic conditions

A 5-μm Spherisorb ODS column (150 mm × 4.6 mm I.D.) (Dalian Institute of Chemical Physics, Academia Sinica, Dalian Chromatographic R & D Centre of China) was used. The column compartment temperature was 40°C. The mobile phase, which was filtered and degassed, consisted of two eluents: solvent A was tetrahydrofuran–methanol–0.1 *M* sodium acetate (pH 7.2) (5:95:900, v/v/v) and solvent B was methanol. The gradient was 100 to 90% A after 1 min, to 80% A after 6 min, to 60% A after 13 min and maintained until 16 min, to 45% A after 20 min, to 0% A after 21 min and maintained until 23 min, and to 100% A after 24 min. The mobile phase flow-rate was 0.6 ml/min.

For detection and identification of the 3-methylhistidine derivative an HP 1046 A programmable fluorescence detector was used at an excitation wavelength of 260 nm and an emission wavelength of 455 nm. The injection volume was 20 μl.

Urine collection and derivatization

Urine collection was started and ended at about 8 a.m. after voiding in the morning. Urine was collected in plastic bottles containing three drops of toluene as a preservative. The urine volume was measured and a 2-ml aliquot was stored at −18°C until analysed.

A urine sample (50 μl) and 400 μmol/l internal stndard (50 μl) were mixed vigorously with acetonitrile (200 μl). Following centrifugation at 13 735 *g* for 10 min, 50 μl of the supernatant were kept until analysis.

The 50-μl supernatant was derivatized by adding a mixture of 50 μl of derivatization reagent and 0.5 ml of sodium borate buffer solution. The reaction was rapid and was completed within 60 s.

RESULTS

There are a variety of other substances apart from amino acids in urine that contain an amino group which can be derivatized with *o*-phthaldialdehyde agent. Fig. 1 shows the chromatogram of the separation under the conditions outlined above. The assay system resulted in a clear separation of 3-methylhistidine derivative from other derivatives.

Urine 3-methylhistidine was determined by internal calibration. The ratio of the peak area of

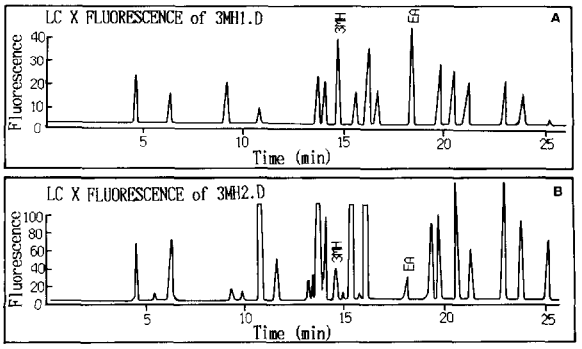

Fig. 1. (A) 3-Methylhistidine (3MH) added to AA-S-18 (amino acid standard solution, Sigma, St. Louis, MO, USA), with ethanolamine (EA) as internal standard. (B) Determination of 3-methylhistidine in urine. Conditions: column, 5-μm Spherisorb ODS (150 mm × 4.6 mm I.D.); eluent A, tetrahydrofuran–methanol–0.1 *M* sodium acetate (pH 7.2) (5:95:900, v/v/v); eluent B, methanol; flow-rate, 0.6 ml/min; gradient, 100 to 90% A after 1 min, to 80% A after 6 min, to 60% A after 13 min and maintained until 16 min, to 45% A after 20 min, to 0% A after 21 min and maintained until 23 min, and to 100% A after 24 min; fluorescence detection, excitation wavelength 260 nm and emission wavelength 455 nm.

3-methylhistidine to that of ethanolamine was calculated daily and compared with the ratio found in biological samples.

A linear correlation between the ratio of 3-methylhistidine to internal standard and the concentration of 3-methylhistidine was found in the range 20.0–100 μmol/l. The slope and intercept of the calibration graph were calculated by least-squares linear regression. The linear regression parameters (correlation coefficients, slopes and *y*-intercepts) exhibited low coefficients of variation over the three-day period. The average of the correlation coefficients exceeded 0.999.

The recovery and within-day and between-day precision of the method of the method were determined and the results are summarized in Table I.

Following trauma, such as an operation on the abdomen, the catabolic response is characterized by a negative nitrogen balance, amino acid disturbance in the serum and intracellular fluid and a moderate loss of weight. It is difficult to avoid a rapid breakdown of body tissue, even if sufficient nutrition is supplied. Since the theory of metabolic support is applied in clinical practice many concepts have been changed in energy supplements, *e.g.*, glucose and fat ratio and amount of nitrogen, but the amino acid formulation of infusions still remains a major problem needing in-depth study.

In this study, 45 gastric cancer patients who had undergone radical total gastroectomy were used as models. The patients were randomly divided into four groups. The control group (n = 12) received ordinary routine infusion after surgery. The other patients received metabolic support with isocaloric infusion, calculated through the Harris and Benedict equation (injury factor 1.5, glucose/fat ratio = 3:1), and isonitrogenous infusion (0.32 g/kg per day). One group (n = 12) received an 8.5% amino acid (AA) infusion and another (n = 8) received a 10% AA infusion. The last group (n = 13) received an 8.5% AA infusion from one to three days after operation, and from four to seven days (flow phase) received

TABLE I

RECOVERY AND WITHIN-DAY AND BETWEEN-DAY PRECISION

	3-Methylhistidine (μmol/l)	Ethanolamine (μmol/l)	Mean recovery (%)	Coefficient of variation (%)	*n*
Within-day	200	400	93.4	7.0	5
	400	400	98.6	5.4	5
Between-day	200	400	97.7	8.7	7
	400	400	95.9	7.5	7

an individual amino acid (IDAA) infusion which was calculated by a self-designed programme according the amino acid serum levels of the patients. The 24-h urine samples were collected from the starting day to seven days during the experiment to determine 3-methylhistidine and other compounds. Table II shows that the metabolic support of IDAA infusion is efficient in reducing 3-methylhistidine excretion and the results may be of benefit to maintaining normality of gastroenteric muscosa so as to avoid bacterial translocation after operation.

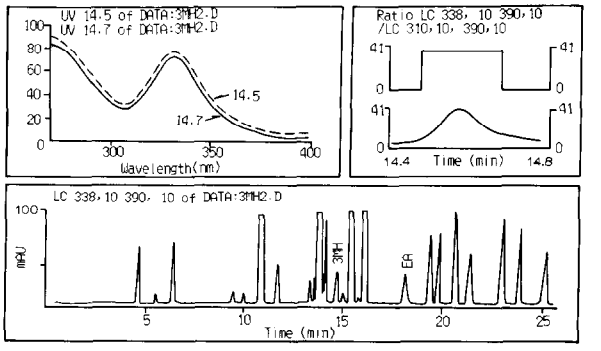

Fig. 2. Ratio plot for the peak purity of 3-methylhistidine in urine. Diode-array detector used at a detection wavelength of 338:10 nm (with 390:10 nm as reference); other conditions as in Fig. 1.

DISCUSSION

Traditionally the identification of a signal separated by HPLC and monitored with a UV–visible detector at a single wavelength is based solely on retention time. However, if a signal is monitored simultaneously at several selected wavelengths, the absorbances can be very different [13,14]. Therefore, it is necessary to analyse the purity of a signal using a diode-array detector.

Accurate determination of urinary 3-methylhistidine requires the separation of 3-methylhistidine from other components in the urine. The optimum separation was achieved by using gradient elution and a 5-μm Spherisorb ODS column (150 mm × 4.6 mm I.D.). Under the specified conditions of solvent and pH, the HPLC signal of 3-methylhistidine standard can be exactly identified at 14.5 min (Fig. 1). Fig. 2 is an example of the use of absorbance ratio data to discriminate the

TABLE II

3-METHYLHISTIDINE EXCRETION IN URINE

Control group (n = 12) received ordinary routine infusion after surgery, 8.5% AA group (n = 12) received 8.5% AA infusion and 10% AA group (n = 8) received 10% AA infusion. IDAA group (n = 13) received 8.5% AA infusion from one to three days after operation, and from four to seven days received individual AA infusion which was calculated by a self-designed programme according the serum levels of amino acids in the patients.

Infusion	Excretion (mean ± S.D.) (μmol/kg per day)							
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Control[a]	5.578	7.45	6.33	7.72	6.43	6.29	6.06	5.92
	±0.237	±0.44	±0.36	±0.43	±0.27	±0.27	±0.27	±0.35
8.5% AA[b]	5.437	7.71	5.45	4.46	4.73	5.34	4.75	4.31
	±0.177	±0.42	±0.32	±0.23	±0.34	±0.32	±0.35	±0.31
10% AA[b]	5.439	7.64	5.44	4.45	4.74	5.36	4.74	4.21
	±0.218	±0.55	±0.37	±0.24	±0.36	±0.33	±0.39	±0.33
IDAA[b,c]	5.449	7.74	5.49	4.42	4.28	4.28	3.92	3.77
	±0.255	±0.54	±0.28	±0.36	±0.20	±0.20	±0.35	±0.21

[a] Compare with the 0 day, $P < 0.05$.

[b] Compare with the control, $P < 0.05$.

[c] Compare with the 8.5% AA or the 10% AA, $P < 0.05$.

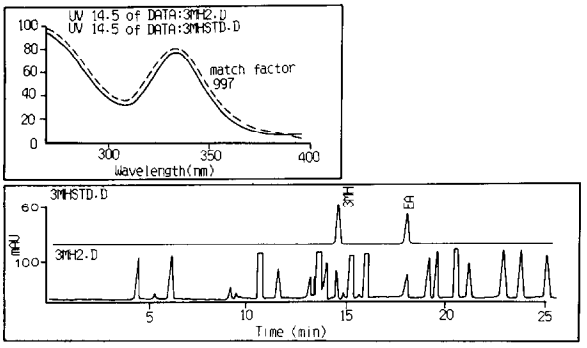

Fig. 3. Comparison of the spectrum of the peak at 14.5 min in urine with that of 3-methylhistidine standard from a standard spectral library. Chromatographic conditions as in Fig. 2.

peak purity at 14.5 min in urine. As the ratios remain constant, the peak is pure.

Fig. 3 illustrates the search with a standard spectral library using a diode-array detector. It shows that the identity match factor of the 3-methylhistidine separated in urine is 997.

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