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Short Communication

High-performance liquid chromatography–electrochemical detection of 3-methylhistidine in human urine

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method is described for the determination of 3-methylhistidine content in human urine using pre-column derivatization with phenylisothiocyanate, isocratic elution with 15 m*M* sodium acetate–acetonitrile (92:8, v/v) and electrochemical detection. The limit of quantitation was 0.1 pmol. The method has been applied in routine analyses of 3-methylhistidine in both clinical and research work.

INTRODUCTION

3-Methylhistidine (3-MHis) is an amino acid that is mainly formed by methylation of histidine in muscle actin and myosin. After release, it is not reutilized for protein synthesis, nor is it involved in other metabolic processes, but it is excreted in urine as 3-MHis (95%) and as N-acetyl-3-methylhistidine (5%) [1]. 3-MHis is an index of muscle protein breakdown and, therefore, of the anabolic/catabolic status, in subjects with normal renal function, where urinary excretion of 3-MHis corresponds with its production [2–4]. 3-MHis is present in all animal muscles. In order to be sure of the endogenous origin of urinary 3-MHis, it is necessary to maintain the patients on a meat-free diet for three days [5,6]. In subjects with chronic renal failure (CRF) this period is much longer [7]. It has been demonstrated that in subjects with normal renal function, 3-MHis urinary excretion is increased after trauma, infections [8–10] and fasting [11]. A correlation between the 3-MHis/creatinine ratio and the nitrogen balance in healthy and stressed premature infants was reported. A ratio <0.2 was associated with a strongly positive balance while higher ratios were associated with a negative or weakly positive nitrogen balance [12].

Numerous studies have been performed to measure the rate of muscle protein degradation from urinary 3-MHis excretion utilizing gas

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chromatography [13,14] or high-performance liquid chromatography (HPLC) with derivatization procedures normally employed for amino acids and fluorescence or UV detection [7,15–20]. Owing to the need to improve sensitivity of the determination we propose modifications of the technique of Sherwood *et al.* [21]. These authors described the determination of amino acids in human blood and urine samples using reversed-phase HPLC after phenylisothiocyanate (PITC) derivatization with amperometric detection. The method, described for quantitation of 40 amino acids, uses gradient elution and a long analysis time (80 min) to resolve the large number of components adequately. The method described in this paper is selective for 3-MHis in human urine. The entire procedure (derivatization and chromatography) is completed with in 30 min.

EXPERIMENTAL

Reagents

Histidine (His), 3-MHis and N-methyllysine (N-MLys) were obained from Sigma (St. Louis, MO, USA). PITC and triethylamine were obtained from Fluka (Buchs, Switzerland). Acetonitrile was of HPLC grade (Carlo Erba, Milan, Italy). All chemicals were of analytical reagent grade and used without further purification. High-purity water was produced with a Milli-Q system (Millipore, Bedford, MA, USA).

Chromatographic system

The HPLC system consisted of a Series 4 liquid chromatograph (Perkin-Elmer, Norwalk, CT, USA) and a Rheodyne Model 7125 injector (Rheodyne, Berkeley, CA, USA) equipped with a 100-μl loop. The column was an Apex phenyl RP (150 mm × 4.6 mm I.D., 5 μm particle size; Jones Chromatography, Mid Glamorgan, UK). Electrochemical detection was performed with a Model 5100 A Coulochem (ESA, Bedford, MA, USA) equipped with a Model 5011 analytical cell containing two porous graphite working electrodes with associated palladium reference electrode. The potentials were set at +0.40 V for the first electrode and +0.80 V for the second electrode. The signal generated by the second electrode was used for the quantitative determination and the chromatograms were analysed with a Chromatopac R4A data processor (Shimadzu, Kyoto, Japan). The mobile phase was a mixture of 15 m*M* sodium acetate–acetonitrile (92:8, v/v) adjusted to pH 6.2 with acetic acid. The flow-rate was set at 1.0 ml/min. During the analysis the mobile phase was recycled.

Standards and controls

Stock standards solutions of 3-MHis in methanol were prepared and stored at −20°C. Urine standards covering the range of 1.5–60 nmol/ml 3-MHis were prepared daily by adding known amounts of the methanolic stock standards to 3-MHis-free human urine; these standards were used to create calibration curves.

Sample preparation

Urine was collected during fourteen days from ten-month-old infant with severe chronic renal failure due to obstructive uropathy in a solitary kidney which needed a urinary diversion and whose urether had been connected to the skin (cutaneous urethero stomy). Urine was collected in a plastic bag. The bag was emptied every 6 h and urine was kept with thymol in a refrigerator at 4°C. At the end of each 24-h period a sample of the whole collection was stored frozen (−20°C).

Derivatization

The PITC reagent was prepared by adding 0.5 ml of PITC to 3.5 ml of methanol. The coupling solvent was made by diluting triethylamine 1:10 with methanol. The solutions were kept at −20°C and were stable for at least six months.

The samples for HPLC were prepared by adding 10 μl of internal standard solution (100 pmol, N-MLys), 200 μl of PITC reagent and 10 μl of coupling solvent to 0.1 ml of urine or standard solution. After incubation at 35°C for 10 min, the solution was evaporated using a rotary evaporator. The dried residue was reconstituted with 0.5 ml of distilled water, mixed with 200 μl of dichloromethane for 1 min with a vortex mixer and centrifuged at 2500 *g* for 3 min in order to remove

excess PITC. Aliquots of the aqueous phase were injected into the HPLC system. According to the literature the phenyl(thiocarbamoyl) (PTC) derivatives are not stable for long periods at ambient temperature [22], therefore they were analysed immediately after derivatization or stored at temperatures below −5°C where they were stable for long periods.

RESULTS AND DISCUSSION

Our method employed isocratic reversed-phase HPLC and the PTC derivatives of 3-MHis were measured by the use of a dual-electrode coulometric detector. The two electrodes were employed in the oxidative screen mode at two different potentials. The potential of the first electrode (V_1) was set at +0.4 V, near the low end of the oxidation current–voltage curves for the PTC derivatives of 3-MHis and N-MLys, to remove compounds having a lower oxidation potential than the analytes. The potential of the second electrode was set at +0.8 V corresponding to the plateau of the current–voltage curves.

Fig. 1A shows a typical chromatogram of a calibration standard containing His, 3-MHis and N-MLys derivatives. Fig. 1B shows the chromatogram of an infant urine sample, containing 3-MHis.

Recovery, reproducibility and detection limit

The recovery of the 3-MHis derivative from urine was calculated by comparing the peak heights observed from urine samples spiked with known amounts of the PCT derivative to those observed from standard solutions (Table I). Standard curves are prepared by plotting the peak-height ratio of the 3-MHis derivative to the internal standard derivative *versus* the known concentration of the analyte in urine over the range 1.5–60 nmol/ml 3-MHis. The regression curve was linear (r^2 = 0.9992). The within-day and between-day coefficients of variation were 2.9 and 3.5% for 20 nmol/ml and 3.5 and 3.9% for 40 nmol/ml 3-MHis in spiked urine.

The detection limit was assessed by injecting decreasing amounts of 3-MHis after derivatization. An amount of 0.1 pmol of 3-MHis could be reliably measured. The signal-to noise ratio was 5:1 in these experiments.

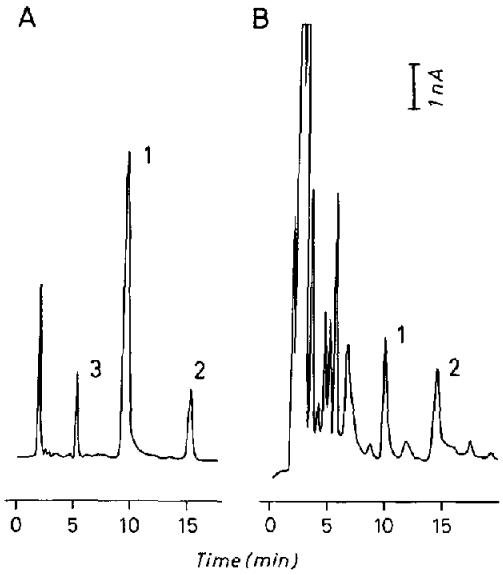

Fig. 1. (A) Chromatogram of a standard solution containing His (2 pmol), 3-MHis (10 pmol) and N-MLys (2 pmol); E_2 = +0.80 V. (B) Chromatogram obtained from urine of an infant with chronic renal failure estimated to contain 4 pmol of 3-MHis. Chromatographic conditions as described in the text. Peaks: 1 = 3-MHis; 2 = N-MLys; 3 = His derivatives.

CONCLUSION AND CLINICAL APPLICATIONS

Our method is simple, sensitive, specific and useful for the routine quantitative analysis of 3-MHis in urine samples. It was applied to evaluate the effect of stress caused by a surgical intervention and the following fasting period on 3-MHis urinary excretion and on the 3-MHis/creatinine ratio in urine of one infant with severe chronic renal failure. 3-MHis excretion increased from 14 to 39 nmol/day in the first three days after surgery, and the 3-MHis/creatinine ratio in urine increased from 0.28 to a peak value of 0.7, thereafter both parameters decreased to the initial values (Figs. 2 and 3). Renal function remained stable during the study. The energy intake (reported as percentage of required dietary allowance) was very low in the first three days after surgery and

TABLE I

RECOVERY OF 3-MHIS AND N-MLYS FROM URINE SAMPLES

The values were calculated by analysing urine samples spiked with standards at six different concentrations. Each value is the mean (± S.D.) of three analyses.

Concentration (nmol/ml)	3-MHis		N-MLys	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
1.63	97 ± 2.7	2.8	105 ± 4.9	4.7
3.26	96 ± 4.3	4.5	98 ± 3.2	3.3
7.50	102 ± 4.8	4.7	101 ± 5.1	5.0
15.00	98 ± 3.6	3.7	93 ± 2.1	2.2
30.00	95 ± 2.1	2.2	99 ± 4.2	4.2
60.00	103 ± 3.4	3.3	97 ± 5.3	5.5

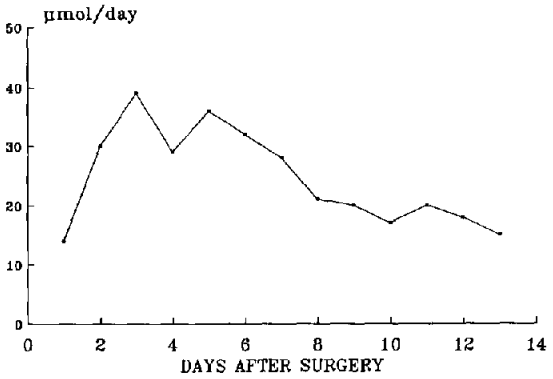

Fig. 2. 3-MHis urinary excretion in one infant with severe chronic renal failure after surgical intervention.

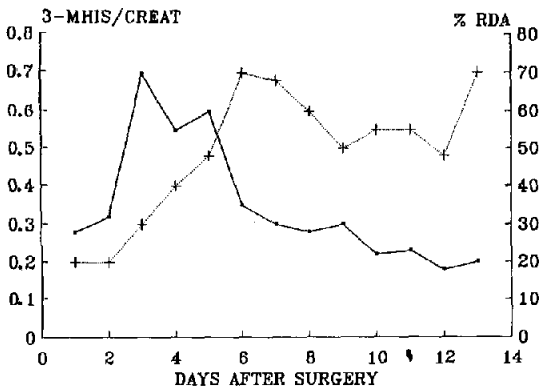

Fig. 3. 3-MHis/creatinine urinary ratio (■) and energy intake (reported as percentage of required dietary allowance, % RDA) (+) in the same patient as reported in Fig. 2.

increased in the following days. This increase correlated well with 3-MHis urinary excretion and the 3-MHis/creatinine ratio. This pattern of 3-MHis excretion is well known in subjects with normal renal function but, to our knowledge, was demonstrated for the first time in an infant with severe chronic renal failure. We believe that urinary 3-MHis excretion can be a reliable index for detecting moderate to severe stress.

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