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Note

Determination of histidine, 1-methylhistidine and 3-methylhistidine in biological samples by high-performance liquid chromatography

Clinical application of urinary 3-methylhistidine in evaluating the muscle protein breakdown in uraemic patients

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Among the amino acids 3-methylhistidine (3-MH) is closely associated with muscle metabolism in the body which originates from degradation of the contractile actomysin system of the muscle [1]. 3-MH is produced in actin of all muscle fibres and in myosin of white muscle fibres by post-translational methylation of peptide-bound histidyl residues [2, 3]. When these proteins are catabolized, free 3-MH is liberated and excreted quantitatively and unchanged in the urine [4—6]. Therefore, the urinary excretion of 3-MH is considered to be an index of the in vivo rate of endogenous myofibrillar protein breakdown [7—9], provided that the individual is maintained on a meat-free diet (i.e. a diet not containing 3-MH) under steady-state conditions.

Young and Munro [7] showed that 80% of the excreted 3-MH in adults comes from actin and the remaining 20% from myosin. Actin and myosin comprise 65% of the total muscle protein and 30—35% of the total body protein pool, thus being the two most abundant proteins in the body.

Unlike 3-MH, little is known about the metabolism of 1-methylhistidine (1-MH) except that it exists in the dipeptide anserine.

Numerous methods have been put forward for the quantitation of 3-MH based on ion-exchange chromatography [10—14], high-performance liquid chromatography (HPLC) [15—18] and gas chromatography [19—21]. These methods generally suffer from lengthy sample preparation and subsequent separation and detection. Furthermore, the methods allow only the determina-

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tion of 3-MH and only in one study [17] could a limited number of other amino acids be quantitated.

In this communication, we present a rapid procedure for the separation of 21 amino acids of protein-free biological specimens by an HPLC method. We have optimized our experimental conditions for the resolution of histidine (His), 3-MH and 1-MH from other amino acids. The method is based on pre-column derivatization of the amino acids with *o*-phthalaldehyde (OPA) in the presence of 3-mercaptopropionic acid (3-MPA); detection was carried out at an excitation wavelength of 340 nm and an emission wavelength of 450 nm. The separation was performed on a reversed-phase column with a two-solvent system. The protocol also allows for the quantitation of tyrosine (Tyr), phenylalanine (Phe) and sixteen other amino acids.

This method of analysis was applied for the quantitation of 3-MH in plasma and urine of uraemic patients kept on a meat-free diet for sixteen days, in order to measure the muscle protein breakdown.

EXPERIMENTAL

Chemicals

Individual crystalline samples of L-amino acids (AMAC standard kit No. 20065) and Brij (30%) were obtained from Pierce Eurochemie (The Netherlands). 1-MH, 3-MH, citrulline, phosphoserine, carnosine, taurine and OPA were obtained from Sigma (St. Louis, MO, U.S.A.). Individual standard stock solutions (1 μM) were prepared in distilled water by the addition of a few drops of 0.1 *M* hydrochloric acid. A standard mixture containing 23 amino acids was prepared to a concentration of 0.1 μM and stored at −70° C until analysed. This standard mixture was diluted as required with distilled water. Methanol was HPLC grade from Rathburn Chemicals (Walkerburn, U.K.). Sodium dihydrogen phosphate, disodium hydrogen phosphate and boric acid were all AnalaR grade, and sodium hydroxide, hydrochloric acid and perchloric acid (70%) in pure form were obtained from Merck (Darmstadt, F.R.G.). 3-MPA was obtained from Fluka (Buchs, Switzerland). Water used for preparation of buffers and standards was deionized and sterile (Milli-Q Water purification system, Millipore).

OPA reagent

OPA (50 mg) was dissolved in methanol (2 ml), to which were added 8 ml of 0.4 *M* borate buffer (pH 10.4 containing 0.6% of 30% Brij). To this mixture, 100 μl of 3-MPA were added. The reagent mixture was kept at 4° C for 24 h before use. The reagent is stable for one week.

Preparation of buffer and the gradient used

Anhydrous Na_2HPO_4 (7.1 g) and $NaH_2PO_4 \cdot H_2O$ (6.9 g) were dissolved separately in 1 l of deionized water to make 0.05 *M* solutions. A buffer of pH 7.2 was made by gradually mixing NaH_2PO_4 with Na_2HPO_4. This buffer is diluted to a concentration of 0.02 *M* with deionized water. For pump A, the mobile phase was 20 m*M* phosphate buffer—tetrahydrofuran (99:1) (solvent A) and for pump B, 20 m*M* phosphate buffer—methanol (30:70) (solvent B) was

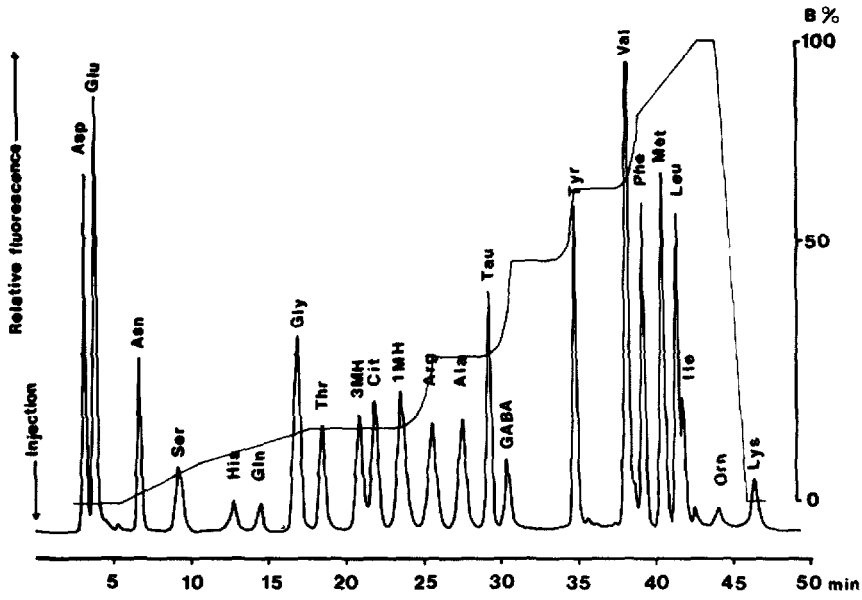

Fig. 1. High-performance liquid chromatogram of standard amino acids (25 nmol/ml) as OPA derivatives. Experimental conditions are given in the text under *Preparation of buffer and gradient used.*

used. Solvents A and B were filtered through 0.45-μm filters, Type HA and HV (Millipore, Bedford, MA, U.S.A.), respectively. The gradient used is shown in Fig. 1. The flow-rate was maintained at 1 ml/min during the run, apart from the first 3 min when the flow-rate was increased linearly from 0.2 to 1.0 ml/min. The column is equilibrated with 100% solvent A for 5 min before and after every run.

Handling of biological samples

A 200-μl volume of a standard mixture of amino acid (100 nmol/ml), urine or plasma was treated with a similar volume of either 30% sulphosalicyclic acid or 1 *M* perchloric acid and centrifuged at 1500 *g* for 15 min. A 200-μl aliquot of supernatant was added to 300 μl of double-distilled water. If not immediately analysed, this solution was stored at −70°C.

The derivatization of OPA with sample was done according to Qureshi et al. [18].

Apparatus

The chromatograph consisted of two solvent delivery pumps, 6000A and M45, a multiple WISP 710B sampler, a data module, a system 730 B controller, and a 420-nm fluorescence detector equipped with an excitation monochromator (340 nm) and an emission cut-off filter (450 nm), all manufactured by Waters Assoc. Separation of amino acids was carried out on a 5-μm Hypersil-ODS column (150 × 4.6 mm I.D.) obtained from Shandon Product (U.K.). A pre-column (50 × 4.6 mm I.D.) obtained from Waters Assoc. was connected to the analytical column. The new analytical column was conditioned first with water—methanol (50:50) and then with 100% solvent A for 1 h each before use.

Patients

Plasma and urine were collected from four patients with chronic renal failure over nineteen days (creatinine clearance < 10 ml/min). The patients were given a diet containing 80 g of meat and providing 40 g of protein per day for the first two days. From the third day, meat was excluded from the diet but the protein intake remained the same, i.e. 40 g per day for the following seventeen days. Plasma samples were collected on the 3rd, 8th, 10th, 12th, 15th, 17th and 19th day. Urine samples were collected over 24 h on the 3rd, 7th, 9th, 11th, 14th, 16th and 18th day.

All samples collected were kept at −70° C until analysis.

RESULTS AND DISCUSSION

3-MPA is recently introduced as an alternative to 2-mercaptoethanol (2-ME) and ethylene glycol (EG), where it has been shown that OPA in the presence of 3-MPA forms more stable derivatives with amino acids according to their fluorescence responses in comparison with 2-ME and EG [22, 23]. The reaction of OPA with amino acid in the presence of 3-MPA is rapid and takes 2—3 min to complete at room temperature. However, the time-dependence factor is proved to hinder the reproducibility of certain amino acids [22]. Thus, using an automatic injector avoids the instability of derivatives with time, which otherwise is observed with a manual injector [24]. Also, the reaction time for OPA and amino acid can be precisely controlled. This procedure shows consistent results with an S.D. of 1—2% over triple analyses for all amino acids.

A typical chromatogram of standard amino acids is shown in Fig. 1, and an analysis of a plasma sample from a uraemic patient is shown in Fig. 2. The whole separation takes less than 1 h. Apart from the quantitation of 3-MH, His

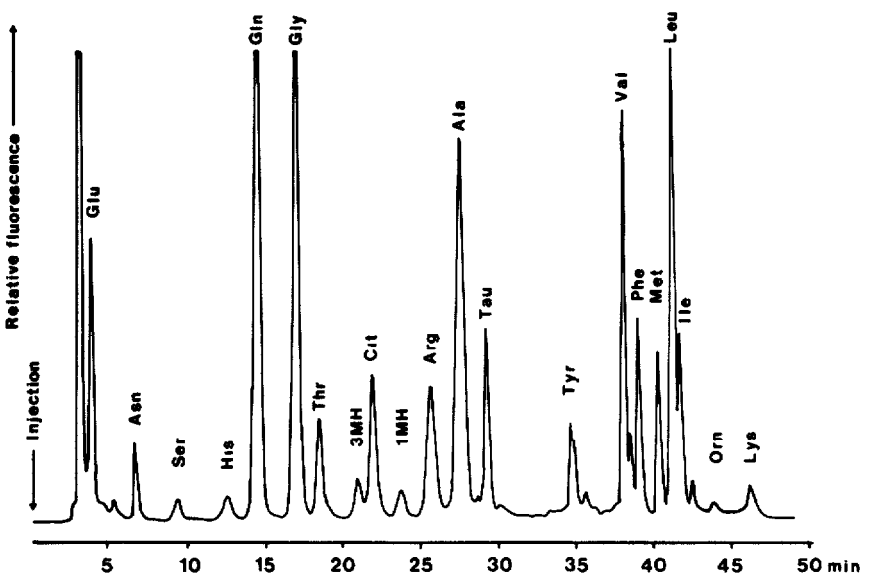

Fig. 2. Separation of amino acids from the plasma sample of a uraemic patient under the conditions of Fig. 1.

and 1-MH, this method also allows quantitation of eighteen other amino acids. As the changes in urinary and plasma levels of several of these amino acids have been correlated with various disease states [25], this method of analysis could be used for diagnosis purposes.

The correlation coefficient (r) between area response and molar concentration in the range 5—100 μmol per 25-μl injection of His, 3-MH, Tyr and Phe is between 0.983 and 0.998.

However, by variety of criteria, this method of analysis appears to adequately resolve 3-MH, 1-MH and His from other amino acids. No preliminary sample

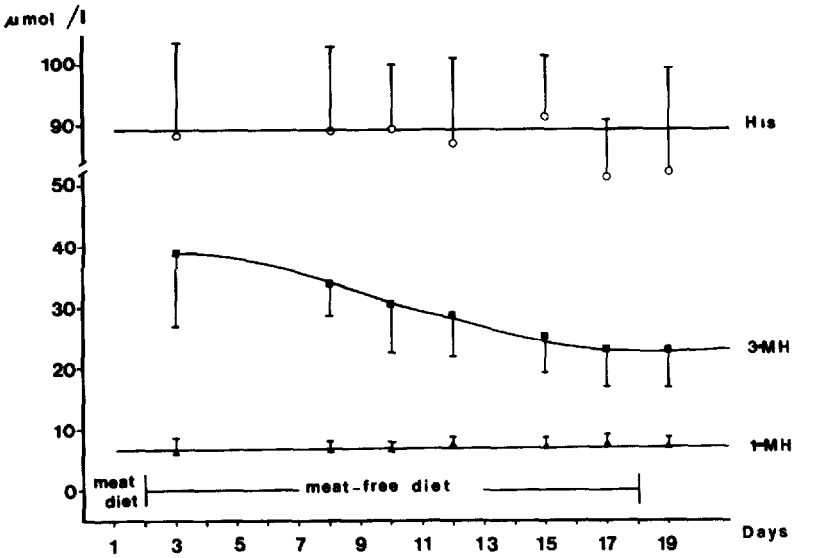

Fig. 3. Plasma concentration of histidine (His), 3-methylhistidine (3-MH) and 1-methylhistidine (1-MH) for a meat-free diet.

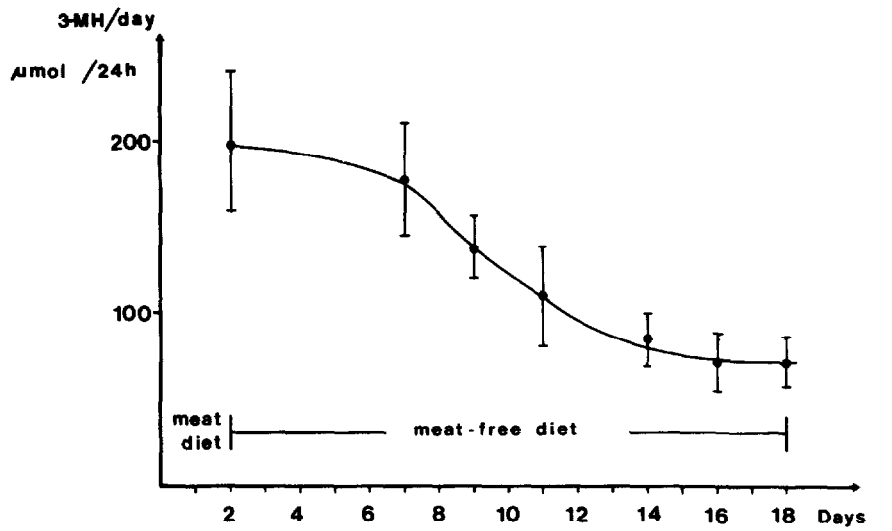

Fig. 4. Urinary excretion of 3-methylhistidine (3-MH) for a meat-free diet.

purification other than deproteinization is necessary. Also, the use of an automatic injector permits reproducible results and eliminates the need for tedious manual injectors which may create variable time factors giving unreliable results when recording the fluorescence intensity of the OPA amino acid adduct.

This method of analysis was applied to the study of four patients with chronic renal failure kept on a meat-free diet for sixteen days. In these four patients, the 3-MH urinary excretion reached a plateau value between the 12th and 14th day of meat-free diet, as shown in Fig. 3. The plasma concentration of 3-MH also showed the same tendency, whereas the concentration of 1-MH and His remained unchanged (Fig. 4). In a previous study [18], it was shown that patients with chronic renal failure kept on a meat-free diet for eight days showed a continuous decrease in plasma concentration and urinary excretion of 3-MH without reaching a plateau value. In healthy individuals, the urinary 3-MH excretion equilibrates within three days under similar conditions [7].

The reason for adopting a prolonged period (twelve to fourteen days) on a meat-free diet in patients with severe renal failure is that the renal clearance of 3-MH is reduced in proportion to the reduction in glomerular filtration rate [26]. This long adaptation period may restrain the use of 3-MH excretion for the evaluation of muscle protein breakdown in such patients.

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