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DETERMINATION OF HISTIDINE AND 3-METHYLHISTIDINE IN PHYSIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

High-performance liquid chromatography based on pre-column derivatization of histidine and 3-methylhistidine with *o*-phthalaldehyde–mercaptoethanol has been used to determine these amino acids in small volumes of plasma and urine. The elution is performed in 45 min on a 5-μm Resolve C_{18} column by a multi-step gradient. The eluted analytes are measured with a fluorescence detector which provides detection limits of less than 1 pmol per 20-μl injection. The correlation between concentration and the integrated area of amino acids gives a linear relationship between 10 and 150 pmol per injection.

Some preliminary results from patients with chronic renal failure under variation of diet are presented.

INTRODUCTION

3-Methylhistidine (3MH) is an analogue of histidine found predominantly in muscle actin and myosin. It is post-translationally formed by methylation of histidine and cannot be reutilized for protein synthesis[1,2]. Therefore, 3MH is a suitable amino acid for monitoring degradation rates of 3MH-containing proteins.

Ion-exchange chromatography has frequently been used for the determination of 3MH in plasma and urine on the basis of post-column derivatization with ninhydrin or *o*-phthalaldehyde (OPA)[3,4], and gas chromatography has recently also been used for this purpose[5].

High-performance liquid chromatography (HPLC) has superseded most of the classical methods, but apart from a few reports[6,7] it has not been used in the monitoring of 3MH in patients with renal failure. We present here a simple and sensitive method to monitor 3MH and histidine in plasma and urine of patients with renal insufficiency. The method is based on the pre-column derivatization of amino acids with OPA in the presence of 2-mercaptoethanol by reversed-phase HPLC.

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MATERIALS AND METHODS

Apparatus

A Waters Assoc. liquid chromatographic system, consisting of two pumps, M6000 and M-45, with multisampler WISP 710B, data module and system controller 730B, was used.

The fluorescence signal was assayed with a filter fluorescence detector Model 420-AC (Waters Assoc.), set at 340 nm and with a 450-nm cut-off filter. The sensitivity was set at 1 μA full-scale.

An analytical column, 5-μm Resolve C_{18} (150 × 4.6 mm i.d.) with a guard column (50 × 4.6 mm I.D.) of same material (both Waters Assoc.) were used for the separation. Before initial use, the column was conditioned with methanol–water (70:30) for 1 h.

Chemicals

Methanol "HPLC" grade was obtained from Rathburn Chemicals (Walkerburn, Scotland). Histidine, 3-methylhistidine and 1-methylhistidine in the L-form were obtained from Sigma. Individual crystalline samples of amino acids were obtained from Pierce (AMAC standard kit No. 20065). Absolute ethanol, boric acid, potassium hydroxide, sodium dihydrogen phosphate 1-hydrate and disodium hydrogen phosphate, (all "AnalaR" grade) were obtained from Merck. Sodium hydroxide, hydrochloric acid and perchloric acid (70%) in pure form were obtained from J. T. Baker. OPA and 2-mercaptoethanol was obtained from Sigma. Brij (30%) was obtained from Pierce.

A standard stock solution (1 m*M*) of tyrosine, phenylalanine, histidine and 3-methylhistidine was prepared in 0.1 *M* HCl. The standard solution was diluted with 0.1 *M* HCl as required.

OPA reagent

Anhydrous OPA (50 mg) was dissolved in 1 ml of methanol. Then 9 ml of 0.4 *M* borate buffer, pH 10.4 (adjusted by addition of 1 *M* potassium hydroxide), containing 0.6% Brij (30%), and 50μl of 2-mercaptoethanol were added. This solution was kept at 4°C for 24 h before use. It remained stable for up to one week, provided after three days 20 μl of 2-mercapthoethanol were added.

Buffer solution

Disodium hydrogen phosphate (7.1 g) and sodium dihydrogen phosphate 1-hydrate (6.9 g) were separately dissolved in 1 l of double-distilled water to make solutions of 0.05 *M*. A pH value of 7.4 resulted when the two were mixed. Fresh buffers were made every day.

Derivatization of amino acids

By using Waters Assoc. automatic sampler with control system, pre-column derivatization was performed automatically with a high reproducibility.

The OPA–2-mercapthoethanol reagent (10 μl) was drawn into the automatic injector, followed by the standard amino acids, plasma or urine sample after treatment with 0.2 *M* perchloric acid (see below under sample preparation section). Both

these solutions were mixed for 2 min in the needle before injection into the column. By this method we obtained reproducible results, with a relative standard deviation of 1–3% for the retention time and peak area of 3MH in first replicate analyses.

Preparation procedure for physiological samples

A 100-μl volume of sample (urine or plasma) was added to 400 μl of 0.2 *M* perchloric acid and centrifuged for 15 min at 1500 *g*. The supernatant was collected and 100 μl were added to 350 μl of methanol. Then 50 μl of amino acid standard (25 mol/ml) containing histidine, 3-methylhistidine, tyrosine and phenylalanine were added to this solution, which was centrifuged again at 1500 *g* for 10 min. The supernatant was collected and kept at 4°C until analysed. Our recovery rate for standard amino acids by this procedure ranged from 91 to 98%.

Chromatographic procedure

The separation was performed using a 5-μm Resolve C_{18} (150 × 4.6 mm I.D.) column with a multi-step gradient system. Mobile phase A consisted of 50 m*M* phosphate buffer (pH 7.4)–tetrahydrofuran–methanol (90:1:9) and mobile phase B was 50 m*M* phosphate buffer–methanol (25:75). The initial condition was 0% B, progressing linearly to 25% B within 15 min. For the next 8 min the linear increase was 3%/min, and for 6 min after that, curve 10 was followed at 4%/min within the next 5 min the proportion of B reached 100%, and this condition was held for 6 min before the proportion of B was reduced to 0% linearly over 4 min. The column was re-equilibrated for 10 min before the next sample was processed. The flow-rate throughout was 1 ml/min and the column temperature was ambient. Solvents in pumps A and B were degassed and filtered through Millipore 0.45-μm filter types HA and HV, respectively. In total 20 μl (10 μl of OPA + 10 μl of standard plasma or urine) were injected into the column.

Plasma and urine samples

Plasma and urine were collected repeatedly from four patients with chronic renal failure (creatinine clearance less than 10 ml/min) over 16 days, with alternate periods of a diet containing 80 g of meat per day (days 1–3 and days 13–16) and a meat-free diet (days 5–12). The dietary content of protein was 40 g/day with either diet. The urine and plasma samples were frozen before being subjected to HPLC analyses.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of OPA–2-mercapthoethanol derivatives of histidine, 3-methylhistidine, tyrosine and phenylalanine (10 nmol/ml) on the 5-μm Resolve C_{18} column.

Fig. 2 shows the separation of plasma sample under our experimental conditions containing 5 nmol/ml of histidine, 3-methylhistidine, tyrosine and phenylalanine.

Fig. 3 shows a standard curve for histidine (His), 3-methylhistidine (3MH), tyrosine (Tyr) and phenylalanine (Phe). Peak areas are linear from 10 to 150 pmol per 20-μl injection with $r^2 = 0.999$.

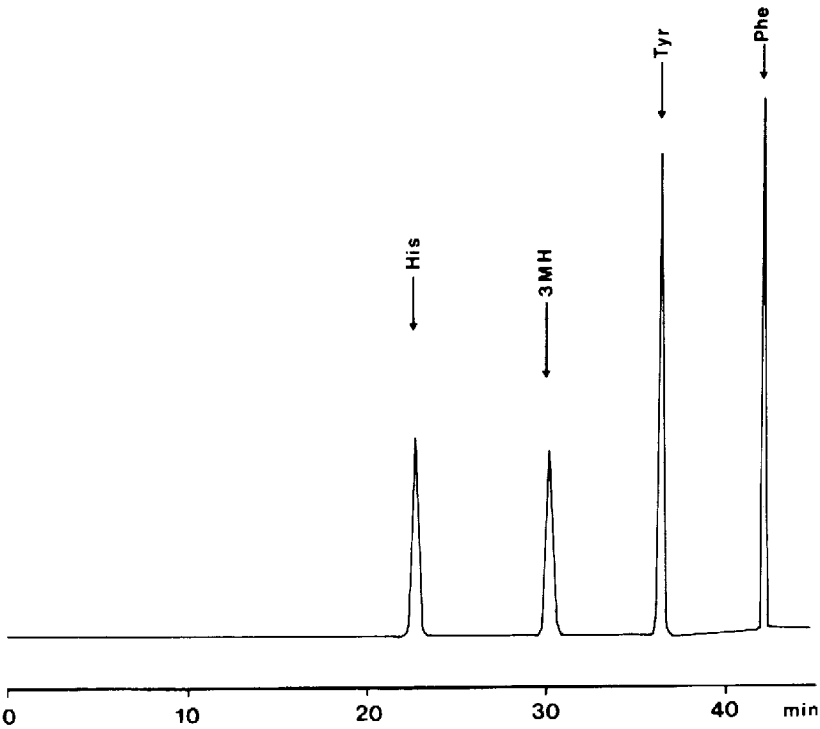

Fig. 1. A chromatogram showing the relative fluorescence and retention times of a mixture of four amino acids as OPA mercaptoethanol derivatives. Each peak represents a 10 nmol/ml concentration of amino acid. For conditions, see Materials and Methods.

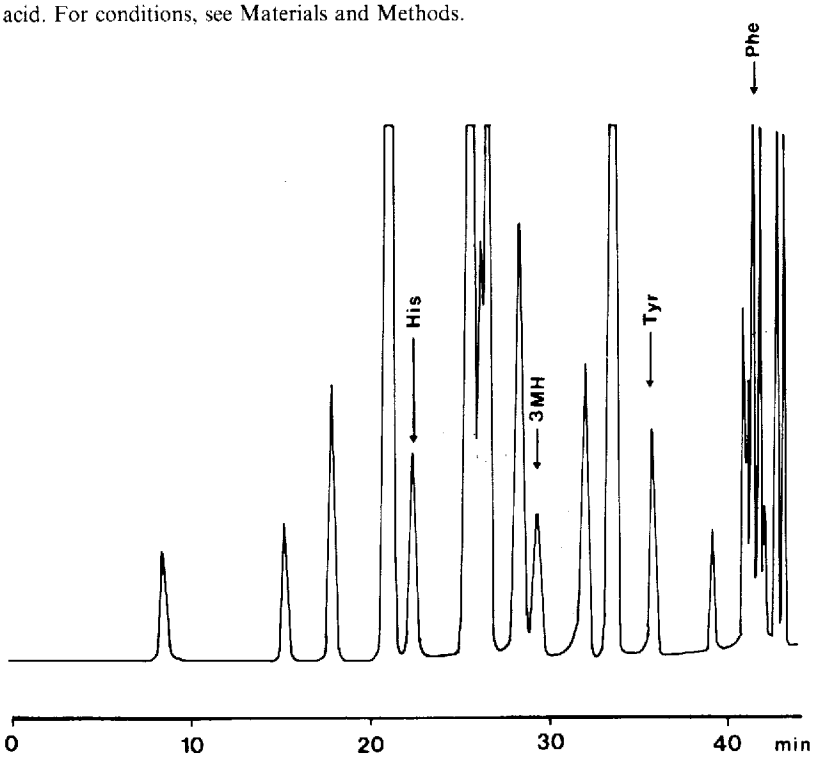

Fig. 2. Separation of amino acids in a urine sample of a uremic patient containing 5 nmol/ml each of His, 3MH, Tyr and Phe, under the same conditions as in Fig. 1.

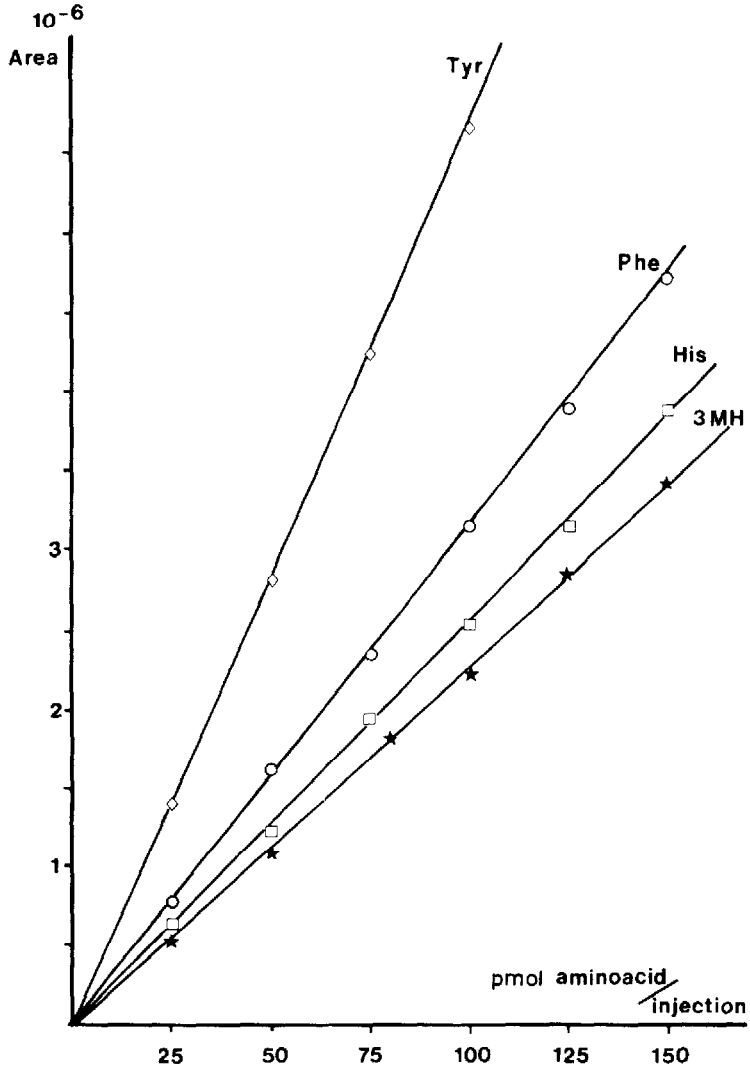

Fig. 3. Standard curves of four amino acids at 10–150 pmol/ml concentration as OPA–mercaptoethanol derivatives *versus* peak area. For conditions see Materials and Methods.

In plasma and urine samples, no amino acid interfered with His, 3MH, Tyr and Phe. When these amino acids were added as internal standards to any of urine or plasma sample in concentrations of from 0–20 nmol/ml, a linear correlation between the amino acids of interest and their areas was obtained (results not shown).

Recently an HPLC method based on the pre-column derivatization of amino acids with fluorescamine was proposed[6] with one solvent system. This system has a 20 min shorter run time than the present method, but can determine only 3MH; His, Phe and Tyr are eluted together with other amino acids. The present method, with a run time of 45 min, is ideal for the precise determination of more than one amino acid.

TABLE I

PLASMA 3MH CONCENTRATION AND URINARY 3MH EXCRETION IN FOUR UREMIC PATIENTS BEFORE, DURING AND AFTER TAKING MEAT-FREE DIET

All samples were chromatographed thrice.

	Day	*Plasma 3MH (μM)*	*Urine 3MH (μmol/24 h)*
Meat	1	–	459 ± 100*
diet	2	88.5 ± 9.2*	378 ± 88
	3	87.8 ± 7.8	321 ± 63
Meat-free	5	91.0 ± 12.0	358 ± 80
diet	6	77.5 ± 7.7	303 ± 65
	8	–	312 ± 65
	9	76.8 ± 14.9	289 ± 65
	10	61.5 ± 9.1	246 ± 49
	11	60.0 ± 9.6	232 ± 34
Meat	12	–	191 ± 30
diet	13	53.0 ± 12.3	–
	15	–	267 ± 33
	16	68.3 ± 8.0	–

* Mean ± S.E.

Clinical application of the method is illustrated in Table I, which presents the plasma 3MH concentrations and urinary 3MH excretion in four patients. It is evident that exclusion of exogenous 3MH supply by providing a meat-free diet gradually reduces the plasma concentration as well as the urinary excretion of 3MH in these patients. In contrast to healthy individuals, in whom urinary 3MH excretion equilibrates within 48 h on meat-free diets, the urinary excretion of 3MH and the plasma concentration continue to decrease over the entire meat-free period in the uremic patients. The markedly reduced glomerular filtration rate and thus a reduced renal capacity to excrete 3MH is probably the reason for this.

These data indicate that the HPLC method based on pre-column derivatization of His and 3MH with OPA 2-mercaptoethanol is reliable for the determination of these free amino acids in urine and plasma. Furthermore, the addition of internal standards considerably increases the accuracy of the analysis. No extraction procedure is necessary apart from treating the sample with 0.2 *M* perchloric acid.

Further studies are in progress to apply this technique to determine 3MH and His in muscles of patients with renal disorders.

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