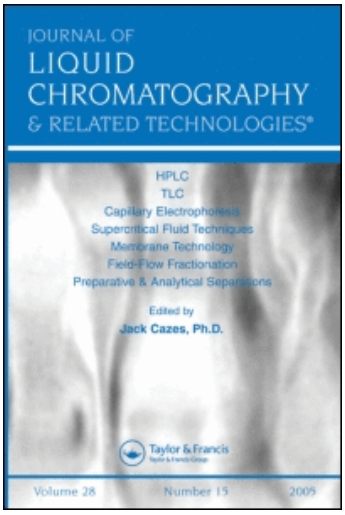

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Improved High Performance Liquid Chromatographic Method for the Quantification of 3-Methylhistidine in Serum and Urine

I. Fermo[a]; E. De Vecchi[a]; C. Arcelloni[a]; P. Brambilla[b]; A. Pastoris[c]; R. Paroni[a]
[a] Istituto Scientifico Ospedale San Raffaele Via Olgettina 60, Milano, Italy [b] Clinica Pediatrica III Universita' di Milano, Italy [c] Ospedale Civile Borgomanero, Novara, Italy

To cite this Article Fermo, I. , De Vecchi, E. , Arcelloni, C. , Brambilla, P. , Pastoris, A. and Paroni, R.(1991) 'Improved High Performance Liquid Chromatographic Method for the Quantification of 3-Methylhistidine in Serum and Urine', Journal of Liquid Chromatography & Related Technologies, 14: 9, 1715 — 1728
To link to this Article: DOI: 10.1080/01483919108049649
URL: http://dx.doi.org/10.1080/01483919108049649

JOURNAL OF LIQUID CHROMATOGRAPHY, 14(9), 1715-1728 (1991)

IMPROVED HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTIFICATION OF 3-METHYLHISTIDINE IN SERUM AND URINE

I. FERMO[1], E. DE VECCHI[1], C. ARCELLONI[1], P. BRAMBILLA[2], A. PASTORIS[3], AND R. PARONI[1]

[1]Istituto Scientifico Ospedale San Raffaele
Via Olgettina 60, Milano, Italy
[2]Clinica Pediatrica III
Universita' di Milano, Italy
[3]Ospedale Civile
Borgomanero, Novara, Italy

ABSTRACT

We optimized an high-performance liquid chromatographic (HPLC) method for the determination of 3-methylhistidine (3-MeHis) in biological fluids. After pre-column derivatization with OPA, analytical separation was achieved on a reversed-phase C18 column by a simple gradient between sodium propionate buffer and acetonitrile. The method is accurate, reproducible and sensitive, and allows the determination of urinary 3-MeHis levels in about 55 min. Other additional 16 amino acids may be easily quantified while the 3-MeHis peak is well resolved from an unknown urinary compound potentially interfering.

INTRODUCTION

Serum 3-methylhistidine (3-MeHis) levels are proportional to the amount of ingested muscular proteins, and therefore may be

considered a useful tool for studies about the pathophysiology of protein absorption in man (1). Moreover, the urinary levels of 3-MeHis are considered a quantitative index of the human skeletal muscle myofibrillar protein breakdown (2-6). In relation to the growing interest about this amino acid, a sensitive, specific, rapid and inexpensive method for the assay in biological fluids is needed.

Because of the complex of urinary matrix and the low levels of 3-MeHis, the analytical technique chosen for its determination should be characterized by an high specificity for this amino acid and should resolve the peak of interest from the whole aminoacidic pattern. At this purpose, a number of techniques have been developed, including the ion-exchange chromatography (7,8), thin-layer chromatography (TLC) (9), gas-chromatography (GC) (10,11), gas chromatography-mass spectrometry (GC-MS) (11,12) and high-performance liquid chromatography (HPLC) (13-19). Most of these methods require sophisticated and not always available instrumentations (GC-MS, amino acids analyzer), and, in the meantime, are not easily introduced in the clinical laboratory routine without the presence of expert and well-trained personnel. Different approaches exist as far as 3-MeHis determination concern. Some authors prefer to purify the amino acid of interest from the urinary matrix with the use of ion-exchange columns before the derivatization and the chromatographic analysis (11,16,17). Some others (13) prefer to destroy anserine (ß-alanylhistidine) and the highly concentrated histidine that may

interfere with 3-MeHis determination. The amino acid analyzer remains however the reference method for this kind of analysis, although very time consuming and expensive (3-MeHis is one of the last eluting in the amino acid pattern).

The HPLC technique with pre-column derivatization, for its versatility and the high selective separation ensured, appeared a very attractive option. In particular, the pre-column derivatizing agent o-phthalaldehyde (OPA) reacts with 3-MeHis to give a fluorescent isoindole that, ensuring a good sensitivity of detection in the pmoles range, allows the determination of 3-MeHis also in serum, where the amino acid is present in very low concentration (4 μM). A number of papers have been published about the use of this technique for amino acid analysis (14-18). We have studied the major parameters affecting the separation of 3-MeHis from the surrounding and interfering components, providing an improved method for its specific quantification in biological fluids. In the same analytical run other 16 amino acids may be simultaneously quantified.

EXPERIMENTAL

Chemicals

The neutral and acid amino acids standard solution, the basic amino acid standard mixture and the internal standard homocysteic acid were purchased from Sigma (St. Louis, Mo, USA). OPA and 2-mercaptoethanol (MCE) were from Pierce (Chester, UK), while iodoacetic acid and propionic acid were purchased from

Janssen Chimica (Beerse, Belgium). All chemicals and solvents were of analytical grade and obtained from Merck (Darmstad, FRG). Water was always doubly distilled and the buffers were filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA) before HPLC analysis.

Reagents and Solutions

OPA (5 mg) was dissolved in 100 μL of methanol, then 0.9 mL of sodium borate buffer (400 mM, pH 9.5) and 4 μL of 2-MCE were added to this solution. Iodoacetic acid (0.75 g) and boric acid (0.6 g) were dissolved in water (100 mL, pH 9.5). 5-Sulfosalicylic acid (SSA) was prepared at 3% (w/vol). The working amino acids standard solution (75 μM) was obtained by mixing 100 μL of the standard physiological solutions (2.5 mM) with 100 μL of glutamine (2.5 mM) and diluting with 3 mL water. Aliquots were stored at -80° C until use. Homocisteic acid was prepared 2.9 mM. Propionic acid (15.68 mL) and anydrous disodium hydrogen phosphate (49.6 g) were dissolved in water under stirring. The solution was exactly titrated to pH 6.50 with few drops of NaOH 2 M, brought to 1 L and stored at room temperature as stock HPLC buffer.

Apparatus

The HPLC was a System Gold Beckman (Palo Alto, CA, USA). The pump mod. 126 was connected through a mod. 406 analogical interface with LS-3 fluorescence detector (Perkin-Elmer, CT, USA). The excitation wavelength was 360 nm and the emission was 455 nm with a fixed scale of 2 for the analysis in urine and of 4 for the analysis in serum.

Chromatographic Conditions

We used a Beckman Ultrasphere ODS (150 X 4.6 mm, 5μm) column with a pre-column Spheri 5 ODS (30 X 4.6 mm, 5μm) (Bronwlee, Santa Clara, CA, USA). Eluent A was 62% water, 30% stock sodium propionate buffer, 8% acetonitrile; eluent B was 45% water, 30% acetonitrile, 25% methanol. The flow rate was 1.4 mL/min. The column was conditioned with 100% of solvent A. At 30 min from the injection the solvent B was increased to 13% over 7 min and held for 18 min. Then B was brought to 90% in 2 min. After 10 min of washing, the solvent A was reported to 100% in 3 min and a new analysis was repeated after other 5 min.

Sample Preparation

To 50 μL of standard solution, urine or serum, homocysteic acid (10 μL) and SSA 3% (100 μL) were added. After centrifugation, 50 μL of the supernatant were transferred in a polypropylene Eppendorf tube containing 100 μL of iodoacetic acid and exactly 1 min after OPA (100 μL) addition, 20 μL were injected into the column.

RESULTS

Urinary and Serum 3-MeHis HPLC Analysis

The analysis of urinary 3-MeHis, because of the presence of many endogenous components, requires very carefully controlled conditions. Anserine (Ans) and carnosine (Carn), dipeptides usually present in physiological and patological biological fluids (19) are the peaks eluting just before 3-MeHis, while ß-alanine

(usually not present in urines) and alanine follow immediately after (Fig. 1). In a first time utilizing a 25 mM sodium propionate buffer concentration (10 %) in the eluent A of the HPLC (Fig. 1/A), we obtained a sufficient resolution of the 3-MeHis peak in respect to the surrounding standard amino acids. When the urinary samples were examined, however, we noted the presence of an unidentified peak not well resolved from 3-MeHis, with a delay in the retention time of 3-MeHis of only 0.5 min (Fig. 1/A*).

Increasing the sodium propionate percentage of the solvent A from 25 mM to 50 and 75 mM (range 10-30%), we observed an improvement in the resolution of 3-MeHis from the unknown peak (1 min and 2.5 min difference in the retention times, respectively) (Fig 1/B* and 1/C*). Therefore to achieve a correct evaluation of 3-MeHis urinary level, avoiding the partial superimposition of the unidentified peak, it is more advisable to apply these last conditions.

However, this interfering compound, affecting the interpretation of 3-MeHis data, was not present when a complete amino acid standard mixture including 35 physiological amino acids was analyzed (Fig. 2).

As the interfering peak was not present in serum, 3-MeHis quantification was performed in this case with eluent A containing only 10% of sodium propionate buffer (Fig.3).

Linearity, Reproducibility and Sensitivity

Quantitative assay was performed by means of the external standard calibration curve, obtained from aqueous solutions of 3-

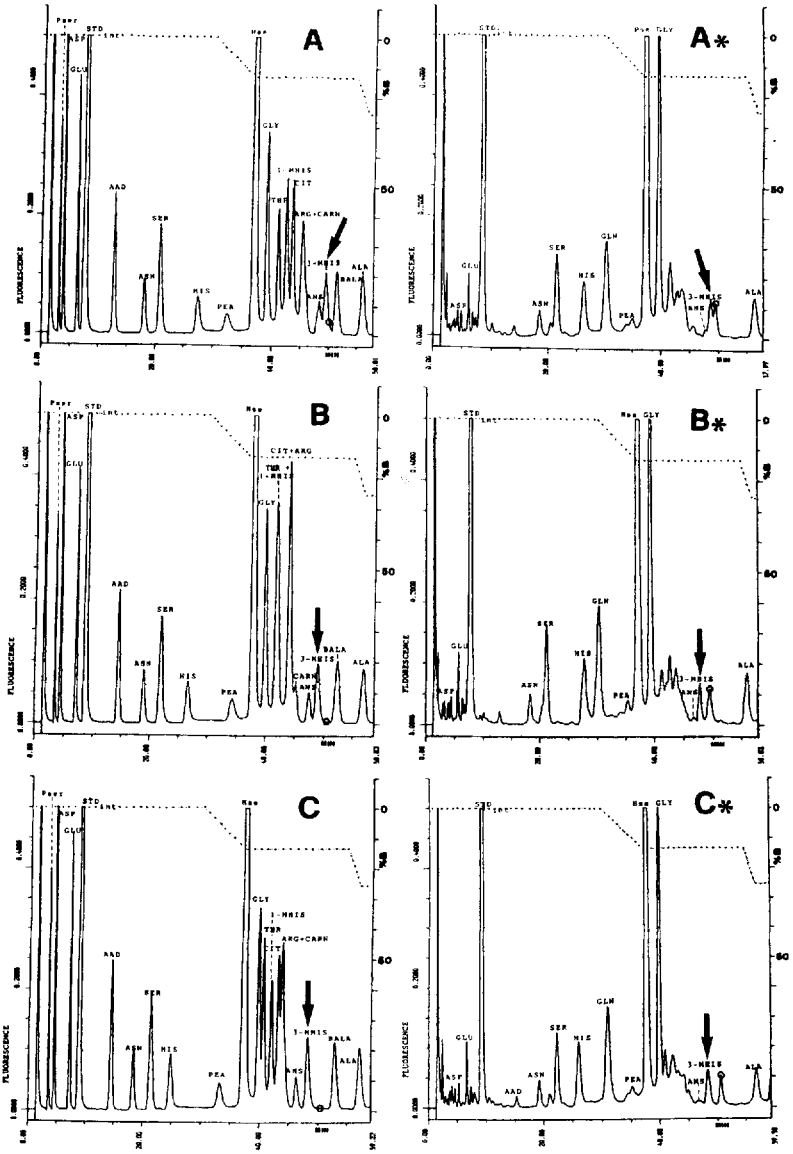

FIGURE 1: Influence of the ionic strength on the OPA-3MeHis separation. Standard mixture (A) and an urine sample (A*), analyzed with 10% sodium propionate in the HPLC eluent A. In B and B* the same samples were analyzed using 20% of sodium propionate. In C and C* analyses were performed with 30% of sodium propionate. The column was a Beckman Ultrasphere ODS (150 X 4.6 mm, 5μm) with pre-column Brownlee cartdrige Spheri 5 ODS (30 X 4.6 mm, 5 μm). Flow-rate 1.4 mL/min, detector with excitation at 360 nm and emission at 455 nm. ○Unidentified peak present in urine specimens. The arrows point out 3-MeHis peaks.

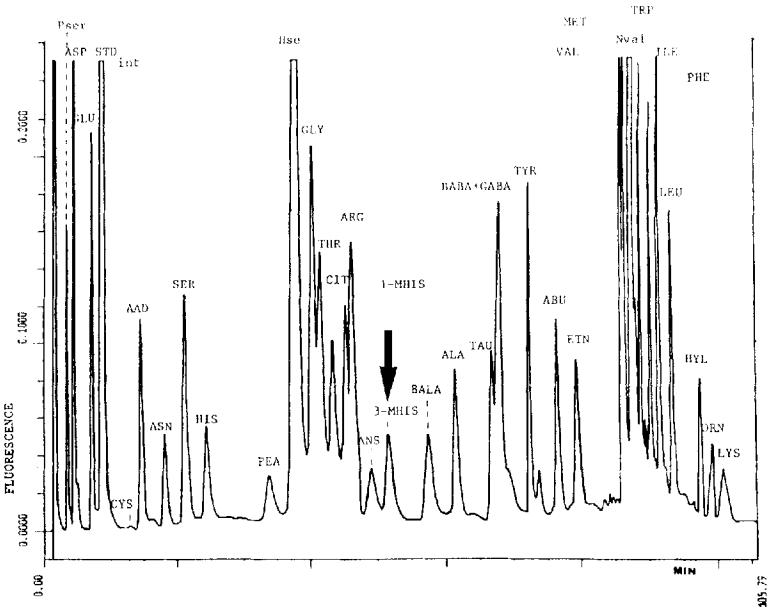

FIGURE 2: Typical chromatographic separation obtained for 35 physiological OPA amino acids (75 μM). Sodium propionate in the eluent A was 75 μM. Other conditions as in fig.1. The gradient utilized is the same reported elsewhere (20).

MeHis in the range 75-1250 μM. The integrated peak areas, normalized to the internal standard, were plotted against the amount of 3-MeHis in the standard solution (Fig. 4). The detector response was linear in all the tested range, the regression data being $Y = 0.00143\ (\pm 0.00007)\ X - 0.021\ (\pm 0.077)$ ($n = 5$) with a correlation coefficient $r^2 = 0.996$.

In order to determine the precision of the assay, 50 μL of the amino acid standard mixture were derivatized and injected (94 pmoles) consecutively ($n = 5$). The within-day coefficient of

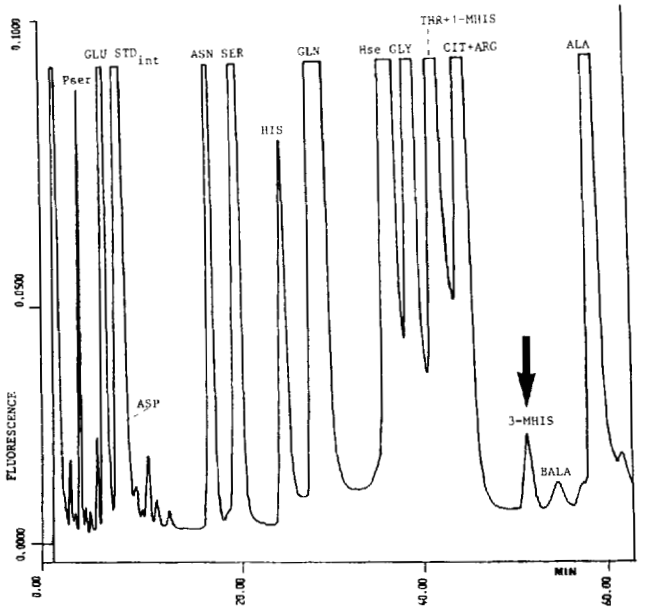

FIGURE 3: Analysis of a serum sample. Sodium propionate in the eluent A was 10%. The detector was set at a fixed scale of 4. The concentration of 3-MeHis was 9 µM (11.2 pmoles injected).

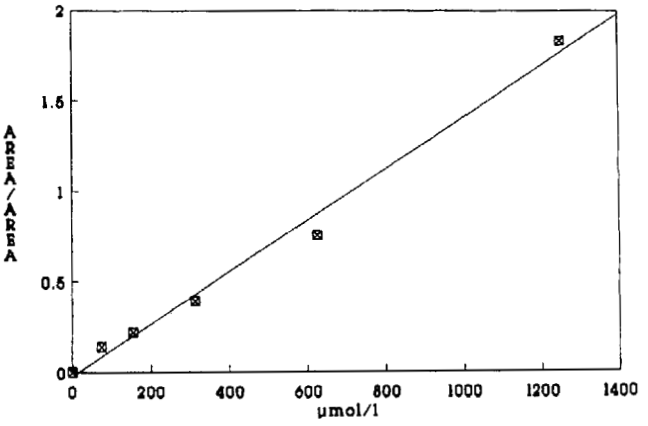

FIGURE 4: Linear regression for the OPA-derivative of 3-MeHis. 3-MeHis/homocysteic acid area ratios were plotted versus the concentration of the standard solutions.

variation (CV %) of 3-MeHis was 3.4%. Testing the same standard mixture on different days (n=7) we obtained a between-day CV% of 6.7. By considering a signal-to-noise ratio of 3, the limit of 3-MeHis detection was 1 pmole injected.

Application

Using the described conditions, we have determined the 3-MeHis urinary excretion in 10 normal-weight healthy children (6-12 years) and in 10 adults (20-40 years) who were placed for three days on a meat-free diet. The 3-MeHis average daily excretion was 119±31 μmol/die for children and 128±24 μmol/die for adults, in good agreement with the literature data (13,16,17).

DISCUSSION

In conclusion the HPLC conditions set up in our study allowed an accurate and sensitive urinary and serum 3-MeHis quantification. The conditions described by Turnell et al. (18) for analysis of OPA-derivatives of amino acids were optimized because of the presence of a urinary unknown compound eluting under the 3-MeHis peak. The intake of the eluent B was delayed and the gradient rate was slowed, while the presence of dimethyl sulfoxide was avoided in order to enhance the column lifetime. By applying a 50 mM HPLC eluent, a satisfactory 3-MeHis quantification was achieved. However, during the lifetime of the analytical column we noted a loss of efficiency with an increasing broadening of the 3-MeHis peak and a decresed resolution from the nearer peaks. By using a 75 mM HPLC buffer a greater symmetry of

the 3-MeHis, usually tailing with the lower ion strenght buffers, was obtained, with a useful column lifetime of about 1500 urine injections and peaks always well formed and clearly resolved. When column back-pressure, the absolute retention times and the peaks shape deteriorated, the replacement of the pre-column was forced. In serum, the interfering peak was absent and 3-MeHis levels were satisfactory determined by using only the 25 mM sodium propionate concentration and applying the same gradient utilized for the urine analysis. It is to point out that the 25 mM buffer allows a better separation of the amino acids group eluting in the middle of the chromatogram (glycine, threonine, 1-methylhistidine, citrulline, arginine), while at the higher propionate concentrations some of these peaks tend to coelute (Fig. 1/B and 1/C).

With the described procedure the 3-MeHis peak elutes at about 52 min, with a remarkable time saving in respect to the traditional amino acid analyzer (19) and in respect to other described HPLC (16, 17) or GC (10, 11) procedures requiring long and tedious pre-purification of the samples. An OPA pre-column procedure for 3-MeHis quantification was previously proposed by Hung et al. (15). However a different elution order of the OPA-amino acid derivatives was reported in the middle part of the chromatogram, with 1-MeHis (usually present in very large amount in urine samples) eluting too close to 3-MeHis peak and precluding an accurate quantification in biological specimens.

We have currently used this method to evaluate the daily urinary 3-MeHis excretion in normal adults and children taken

under a meat-free diet for three days, obtaining a good concordance with the references values (13,16,17). In addition, with the described analytical procedure we have quickly determined the 3-MeHis excretion in 20 obese children taken under a low calories controlled diet for a six month period. The complete data from this clinical study will be published elsewhere.

ACKNOWLEDGEMENTS

This research was partially supported by CNR (Consiglio Nazionale delle Ricerche). We thank Miss Marilena Lomartire for her valuable technical assistance.

REFERENCES

1. Bachmann, K., Galeazzi, R., Millet, A. and Burger A., Plasma levels of 3-methylhistidine after ingestion of the pure amino acids or of muscular proteins measured by radioimmunoassay, Metabolism, 33, 107, 1984.

2. Asatoor, A.M. and Armstrong, M.D., 3-Methylhistidine, a component of actin, Biochem. Biophys.Res. Com., 26, 168,1967.

3. Long, C.L., Haverberg, L.N., Young, V.R., Kinney, J.M., Munro, H.N., and Geiger, J.W., Metabolism of 3-methylhistidine in man, Metabolism, 24, 929, 1975.

4. Haverberg, L.N., Deckelbaum, L., Bilmazes, C., Munro, H.N., and Young,V.R., Myofibrillar protein tournover and urinary N-methylhistidine output, Biochem. J., 152, 503, 1975.

5. Munro, H.N., Young, V.R., Urinary excretion of N-methyilhistidine (3-methylhistidine): a tool to study metabolic responses in relation to nutrient and hormonal status in health and disease of man, Am. J. Clin. Nutr., 31, 1608, 1978.

6. Bilmazes, C., Uauy, R., Haverberg, L.N., Munro, H.N. and Young V.R., Muscle protein breakdown rates in humans based on N-methylhistidine (3-methylhistidine) content of mixed proteins in skeletal muscle and urinary output of N-methylhistidine, Metabolism, 27, 525, 1978.

7. Lukaski, H. and Mendez, J., Relationship between fat-free weight and urinary 3-methylhistidine excretion in man, Metabolism,29, 758, 1980.

8. Calles-Escadon, J., Cunningham, J., Snyder, P., Jacob, R., Huszar, G., Loke J. and Felig, P., Influence of exercise on urea, creatinine and 3-methylhistidine excretion in normal human subjects, Am. J. Physiol., 246, E334, 1984.

9. Monboisse J.C., Pierrelee P., Bisker, A., Pailler, V., Randoux, A. and Borel, J.P., Quantitation of the urinary methylhistidine isomers by a combination of thin-layer and fluorimetric techniques, J. Chromatogr., 233, 355, 1982.

10. Vielma, H. and Mendez, J., Gas-chromatographic separation of 3-methylhistidine, J. Chromatogr., 196, 166, 1980.

11. Marcucci, F., Colombo, L. and Mussini, E., Gas-chromatographic quantitative determination of 1- and 3-methylhistidine in urine and muscles: comparison with glass capillary determination, J. Chromatogr., 336, 356, 1984.

12. Matthews, D.E., Starrem, B., Drexler,A.J., Kipnis, D.M. and Bier D.M., Picomole assay for N-methylhistidine by gas chromatography-mass spectrometry, Anal. Biochem., 110, 308, 1981.

13. Ward, L.C., Miller, M. and Hawgood, S., Simple and high-performance liquid chromatographic method for the quantification of 3-methylhistidine, J. Chromatogr., 223, 417, 1981.

14. Hancock, W.S. and Harding D.R.K., Rapid analysis of 3-methylhistidine in urine, plasma, muscle and amniotic fluid with a single high-performance liquid chromatographic system but with different ion-pairing reagents, J. Chromatogr., 228, 273, 1982.

15. Hung, S.S.O. and Moon, T.W., Determination of free methylhistidine and amino acids by high-performance liquid chromatography, J. Chromatogr., 269, 114, 1983.

16. Minkler, P.E., Ingalls, S.T., Griffin, R.L. and Hoppel C.L., Rapid high-performance liquid chromatography of 3-methylhistidine in human urine, J. Chromatogr., 413, 33, 1987.

17. Caccialanza, G., Gandini, C., Kitsos, M., Ponci, R. and Benzos, M., Determination of urinary 3-methylhistidine by high-performance liquid chromatography, J. Pharmaceut. Biomed. Anal., 6, 1055, 1988.

18. Turnell, D.C. and Cooper, J.D.H., Rapid assay for amino acids in serum or urine by pre-column derivatization and reversed-phase liquid chromatography, Clin. Chem., 28, 527, 1982.

19. Murayama, K. and Sugawara, T., Determination of carnosine and homocarnosine in biological materials, J. Chromatogr., 224, 315, 1981.

20. Fermo, I., De Vecchi, E., Diomede, L. and Paroni, R., Serum amino acid analysis with pre-column derivatization: comparison of the o-phthaldialdehyde and N,N-diethyl-2,4-dinitro-5-fluoroaniline methods, J. Chromatogr., 1990 (in press).

Received: November 29, 1990
Accepted: January 15, 1991