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RAPID ION-EXCHANGE METHOD FOR THE DETERMINATION OF 3-METHYLHISTIDINE IN RAT URINE AND SKELETAL MUSCLE

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SUMMARY

A method for the determination of 3-methylhistidine using an automatic amino acid analyser has been developed. A single column system with lithium buffer (pH 3.950, 0.500 mol/l lithium and 0.067 mol/l citrate) was used for elution. The standard amino acid mixture of basic amino acids and some dipeptides usually present in physiological fluids was analysed for the development of the method. 3-Methylhistidine eluted in 46.7 ± 0.049 min and the peak area coefficient of variation for the same sample was 1.07%.

As 3-methylhistidine is completely resolved from the other basic amino acids and some dipeptides (anserine and carnosine), this method is suitable for the analysis of urine and muscle extracts as well as skeletal muscle protein hydrolysates where this amino acid is present in much lower concentrations than other amino acids.

INTRODUCTION

3-Methylhistidine (3-MeHis) is a constituent of skeletal muscle proteins [1]. In other tissues it is present in negligible quantities [2]. As this amino acid is formed by post-translational methylation of peptide-bound histidine [3,4], the rate of its urinary excretion has become a valuable catabolic marker of the skeletal muscle proteins and has increasingly been used for this purpose in recent time [5—8].

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There are many methods for the estimation of 3-MeHis based on ion-exchange chromatography [9—11] or high-performance liquid chromatography (HPLC) [12—15]. Most of them have been developed for urine determinations [9—14]. Some of them have been tested on plasma [14, 19] and muscle [15,17] samples. It seems that there are different problems if various biological samples are analysed. Some workers, using HPLC systems, have employed different ion-pairing reagents for different biological samples [15]. Pyridine elution for muscle protein hydrolysates has been recommended by some authors [16]. Determination of 3-MeHis in the skeletal muscle proteins could be a problem as this amino acid is present in much lower concentrations than the others [17]. If the free form of this amino acid has to be measured in muscle samples, anserine and carnosine could be a problem because of their similar chromatographic characteristics.

This report describes an ion-exchange chromatographic method for the determination of 3-MeHis that overcomes these problems and is suitable for both urine and skeletal muscle samples. It needs neither different analytical conditions for different biological samples nor special samples preparation or derivatization required when other methods are used [12—16]. Besides, it is faster than many methods based on ion-exchange chromatography [18—21], sufficiently sensitive for urine and muscle protein hydrolysate samples and more precise compared to the other methods [9,12,13].

EXPERIMENTAL

Equipment

Analyses were performed using a Model 121 M automatic amino acid analyser (Beckman, Palo Alto, CA, U.S.A.) operated according to the flow diagram presented in Fig. 1. The single-column system using the short column of the instrument packed with ion-exchange resin was employed. Reagent flows were regulated with pumps and the temperature of the chromatographic column maintained by a circulating water bath. Analyser operating conditions are summarized in Table I.

Amino acids eluted from the chromatographic column were detected with a ninhydrin reagent. The absorbance was measured at 570 nm using a flow cuvette and recorded by a two-channel recorder. The channel operating at the highest sensitivity was connected to a calculator; another channel operated at the usual sensitivity of the instrument. The calculator (Beckman, Data 126 System Calculator) was used for the determination of elution times (in minutes), peak area measurement (absorbance converted to the digital signal) as well as for the quantification of separated compounds.

All functions of the analyser were programmer-controlled. The programmer has 42 channels and performs different functions at predetermined times (pumps, transfer and injection of the samples, control buffer selector valves, control of column temperature, etc.). The instrumental analysis sequence is given in Table II and calculator run parameters in Table III.

Chemicals

All chemicals were of analytical grade (Merck). Water for preparing buffers

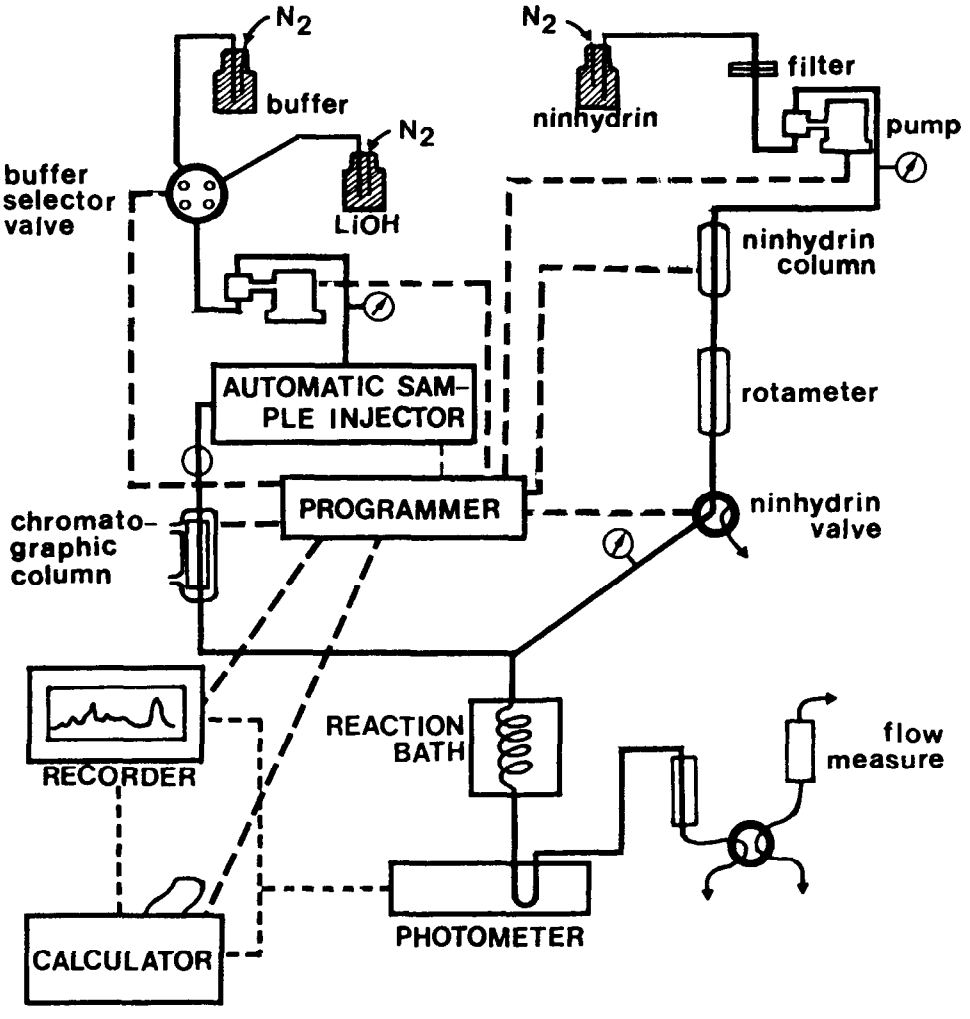

Fig. 1. Flow diagram of analytical system and programmer control.

TABLE I

ANALYSER OPERATING CONDITIONS

Ion-exchanger	AA-10 resin (Beckman)
Column diameter	0.28 cm
Column length	13.0 cm
Resin bed height	4.5 cm
Buffer: pH	3.950 ± 0.010
Lithium	0.500 mol/l
Citrate	0.067 mol/l
Buffer flow-rate	7.5 ml/h
Ninhydrin flow-rate	3.5 ml/h
Column temperature	64°C
Photometer: wavelength	570 nm
absorbance range	0.1 a.u.
Volume of the applied sample on the column	50 μl and 100 μl

TABLE II

INSTRUMENTAL ANALYSIS SEQUENCE

Step No.	Step time (min)	Operation
1	20.0	Equilibration before automatic run start; buffer and ninhydrin to the column
2	0.1	Automatic run start
3	2.0	Sample transfer from the coil on the automatic sample injector plate to the metering loop
4	0.1	Calculator on
5	1.0	Sample injection on the column
6	52.0	Chromatographic analysis
7	2.0	Regeneration of ion-exchange resin; lithium hydroxide to the column
8	15.0	Equilibration of the resin with the buffer
9	5.0	Calculator off, results printing
10	0.1	Programmer chart to step No. 2 for next sample analysis

TABLE III

CALCULATOR RUN PARAMETERS

PW	(peak width)	20
SS	(slope sensitivity)	1000
BL	(baseline test)	5
ML	(maximum baseline level)	50000
MA	(minimum area)	1000
FP	(number of fused peaks)	255

was ion-exchanged and sterile (Milli-Q Water Purification System, Millipore).

Lithium citrate buffer pH 3.950 ± 0.010 (23°C) with 0.500 mol/l lithium and 0.067 mol/l citrate was used. It was prepared by dissolving citric acid monohydrate and lithium hydroxide in water, and was then titrated to the desired pH value with concentrated hydrochloric acid and brought to final volume with water. The buffer was filtered before use (Millipore 0.2 μm mesh). Octanoic acid was used as a preservative (0.1 ml per 1000 ml).

Lithium citrate buffer (0.150 mol/l lithium and 0.050 mol/l citrate) pH 2.2 was used for dilutions. The buffer was filtered (Millipore, 0.2 μm mesh).

AA-10 resin (Beckman, PN 338013) was used. Lithium hydroxide solution (0.3 mol/l) was used for resin regeneration.

Dimethyl sulphoxide—ninhydrin reagent (Beckman, PN 336452) was used for amino acid detection. This reagent was mixed with a premeasured amount of hydrindantin according to the directions given.

The basic amino acid solution (Hamilton, Type P-B, PN 77729) contained 2.5 μmol/ml lysine, arginine, histidine, 1-methylhistidine, 3-methylhistidine, hydroxylysine, γ-aminobutyric acid, ornithine, ethanolamine, 15.0 μmol/ml creatinine, 2.5 μmol/ml carnosine and 1.25 μmol/ml anserine. This solution was diluted 25 times with a sample diluting buffer, pH 2.2 (solution A). Solutions of individual compounds were prepared from standard substances (Serva) by dissolving them in the sample diluting buffer.

Samples

Rat urine was analysed and as the rat excretes 3-MeHis mostly in the acetylated form [4], the urine was hydrolysed in hydrochloric acid (6 mol/l) at 110°C for 20 h, then evaporated and dissolved in the sample diluting buffer.

The rat muscle (m. gastrocnemius) was extracted in sulphosalicylic acid (0.16 mol/l). The deproteinized extract was brought to pH 2.2 with lithium hydroxide and to the final volume with the sample diluting buffer.

Mixed rat muscle protein hydrolysates (m. gastrocnemius) were prepared according to the method of Haverberg et al. [2].

TABLE IV

ELUTION TIMES OF 3-METHYLHISTIDINE AND SOME OTHER COMPOUNDS

Compound	Elution time (min)
Tryptophan	23.9
Hydroxylysine	29.5
Ammonia	33.3
Creatinine	33.6
Ornithine	35.6
Lysine	36.0
Histidine	41.3
1-Methylhistidine	41.6
3-Methylhistidine	46.7
Carnosine	56.9
Arginine	62.5

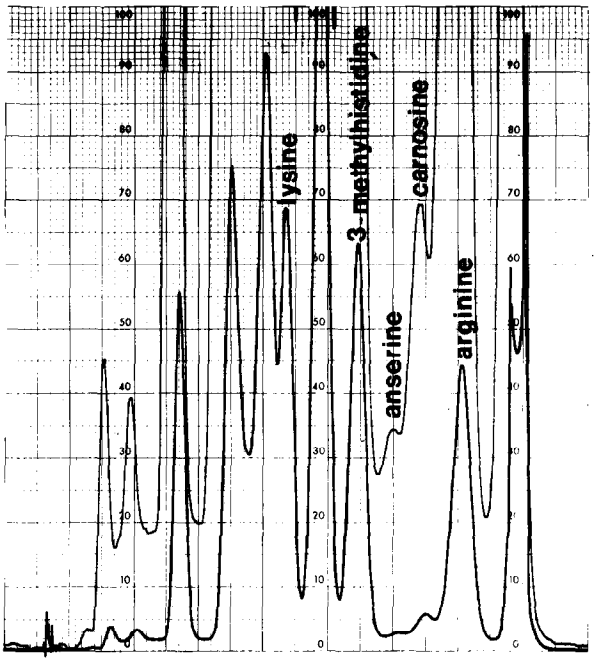

Fig. 2. Chromatogram of standard amino acid mixture (solution A). The mixture was injected into the column in 100 µl of lithium citrate dilution buffer and contained 100 nmol/ml of each compound with the exception of anserine (50 nmol/ml).

RESULTS

Elution times of the compounds examined were determined by their separate injection and elution from the chromatographic column. These results are presented in Table IV.

To test the interaction between compounds in the mixture and to determine the precision of 3-MeHis measurement, a standard calibration mixture (solution A) was analysed. The chromatogram obtained with this mixture is shown in Fig. 2. As can be seen, lysine, 3-MeHis, arginine as well as anserine and carnosine are resolved as separate peaks.

The sensitivity of 3-MeHis determination was also tested. A calibration curve was obtained using solutions with increasing concentrations of this amino acid (from 10 to 300 nmol/ml) and it was found to be linear for concentrations ranging from 10 to 150 nmol/ml.

TABLE V

REPEATABILITY OF ELUTION TIMES AND PEAK AREAS OF SEPARATED COMPOUNDS (n = 5)

Compound	Concentration (nmol/l)	Elution time			Peak area (calculator readings)		
		Mean (min)	S.D. (min)	C.V. (%)	Mean	S.D.	C.V. (%)
3-MeHis	100	46.7	0.049	0.105	9,947,375.4	106,238.0	1.068
Arginine	100	62.5	0.049	0.078	8,668,142.0	55,302.7	0.638
Lysine	100	36.0	0.040	0.111	9,055,923.6	141,906.3	1.567
Anserine	50	52.7	0.040	0.076	137,484.5	6,709.2	4.880
Carnosine	100	56.9	0.075	0.131	615,251.0	12,489.6	2.030

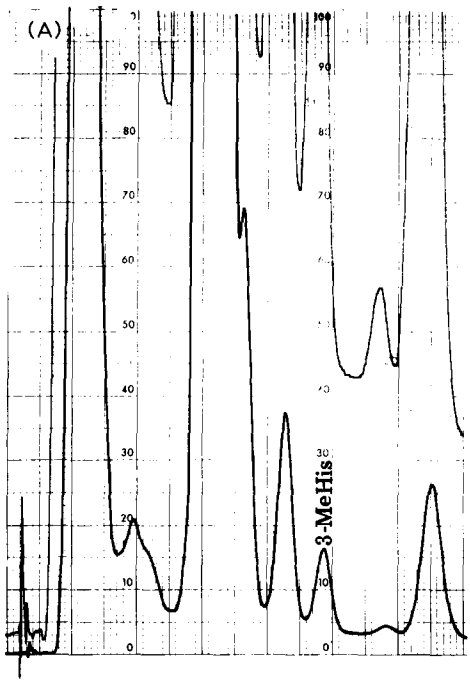

Fig. 3.

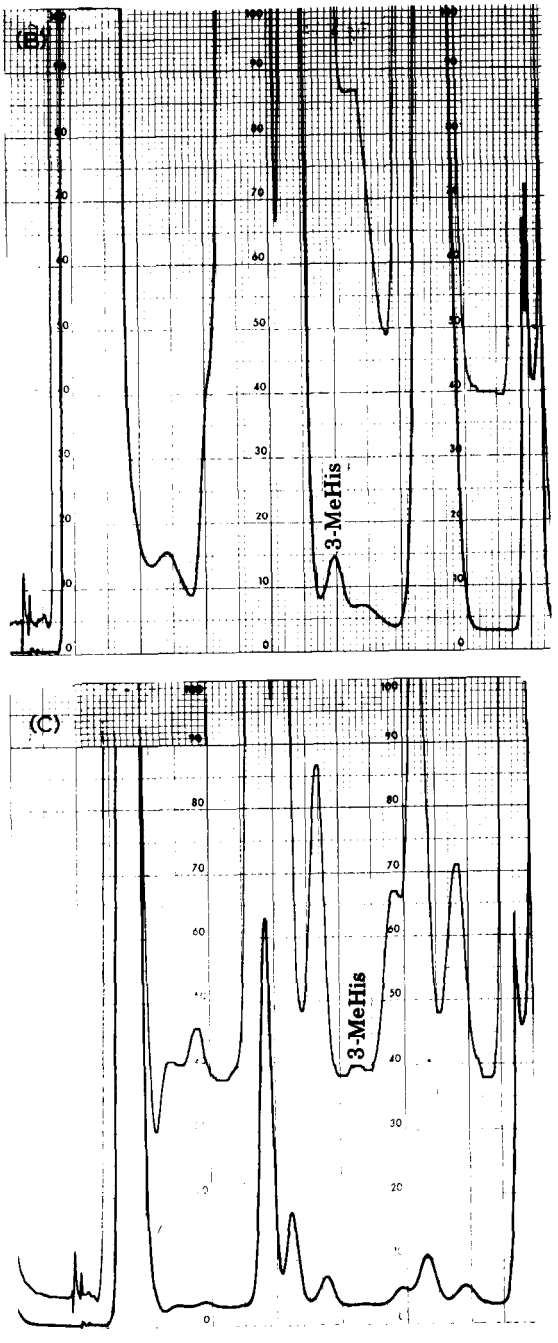

Fig. 3. Chromatograms obtained with rat urine (A), muscle protein hydrolysate (B) and muscle extract (C), illustrating good resolution of 3-MeHis from the other compounds; 50 μl of each sample were applied on the column.

The statistical analysis of the peak areas and elution times obtained for compounds completely separated from the mixture are presented in Table V. There are small variations in elution times and the calculator always identifies

the peaks with good precision. The position of 3-MeHis on the chromatograms ranged from 46.7 to 46.8 min (C.V. = 0.1%) and the peak areas obtained in replicate with the same standard solution had a very low coefficient of variation (C.V. = 1.07%).

Applicability of this method for 3-MeHis estimation in physiological fluids was tested using muscle extract and protein hydrolysates as well as urine samples. Recovery of the added 3-MeHis to muscle extract was 99.4%. In the samples examined this amino acid eluted as a separate peak (see Fig. 3).

DISCUSSION

The separation of 3-MeHis from other basic amino acids was complete under the descirbed analytical conditions. Lysine and arginine as well as anserine and carnosine were resolved (Fig. 2) and could be quantitated by this method. At the same time, 1-MeHis coeluted with histidine (His). The other amino acids eluted together or before ammonia.

As 3-MeHis is separated from the basic amino acids as well as from some dipeptides (anserine and carnosine) present in muscles, this method is suitable for the determination of 3-MeHis in muscle extracts without preliminary removal of peptides (Fig. 3). In addition, since the 3-MeHis peak is sufficiently far from the His/1-MeHis peak, the method can be used with good accuracy for the determination of 3-MeHis in muscle protein hydrolysates where this amino acid is present in much lower concentrations than other amino acids (Fig. 3). It seems that with other methods [16,17] the determination of 3-MeHis in muscle protein hydrolysates could be a problem because the time of its elution from the chromatographic column is too close to histidine. For the determination of 3-MeHis in skeletal muscle protein hydrolysates by the present method, neither special preliminary sample preparation nor pyridine elution, as recommended by some authors [16], is needed.

The method that we have developed for the determination of 3-MeHis is faster than some other methods based on ion-exchange chromatography [18–21]. It is as fast as the method recommended by Long and Geiger [10] but the elution time is achieved with ten times lower flow-rates of buffer and ninhydrin. Although some methods for 3-MeHis based on HPLC are faster [12–14], duration of analysis is rather similar since sample preparation is longer. At the same time, this present method can be used for the determination of lysine, arginine, anserine and carnosine. While this method for 3-MeHis is as sensitive as some other methods [9], its precision is much better compared to those based either on ion-exchange chromatography [9] or on HPLC [12,13]. Repeatabilities of peak location (C.V. = 0.1%) and peak areas (C.V. = 1.07%) permit a precise determination of 3-MeHis concentrations.

We examined the influence of some working parameters on the 3-MeHis elution rate from the column and on its resolution from other compounds. It is very sensitive to column temperature changes. A temperature decrease produced a slower elution of this amino acid and of the other amino acids from the column. At the same time, lowering of the temperature resulted in an increased distance between anserine and carnosine. At 33°C these peptides eluted very slowly producing wide peaks. At the same temperature 3-MeHis

eluted in 72.7 min, at 41°C in 64.7 min, while at 67°C it eluted in 43.7 min. However, with the temperature higher than 64°C, the peaks were too close and their determination was less precise.

The buffer composition affected the 3-MeHis elution time and its resolution from other amino acids. While the pH of the buffer was unchanged (pH 3.95), change in ionic concentration (1 mol/l lithium and 0.166 mol/l citrate) produced a faster 3-MeHis elution from the column. However, this buffer did not resolve anserine, carnosine and arginine, which eluted together, while the 3-MeHis and His/1-MeHis peaks were too close. A buffer flow faster than recommended produced similar changes in the distance between the 3-MeHis and His/1-MeHis peaks. Therefore, the conditions presented at the beginning of this paper were chosen as optimal.

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