Journal of Chromatography, 187 (1980) 271–276

CHROM. 12,313

Note

Chromatographic method for the measurement of hydroxylysine, hydroxylysine glycosides and 3-methylhistidine in human urine

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(First received May 22nd, 1979; revised manuscript received August 14th, 1979)

Our laboratory has been interested in measuring collagen breakdown products excreted in the urine of individuals exposed to inhalation of toxic gases[1,2], of individuals who have spent long periods of time in space[3] and of a wide variety of patients considered to have diseases affecting the turnover of collagen and basement membrane[3,4]. Since in several of these conditions considerable loss of weight may occur, we have been interested also in measuring the urinary excretion of 3-methylhistidine (3-Mehis). This amino acid is methylated at the peptide level in the course of post-ribosomal modifications of muscle proteins[5], it is not recycled and its excretion is considered to be a suitable indicator of muscle proteins turnover[5].

Although several excellent methods have been published for the measurement of total hydroxylysine (Hyl), free Hyl and Hyl glycosides in alkaline and acid hydrolysates of collagen[6–12], we have experienced some difficulty in applying those methods for analyses of urine samples, because of the abundance in human urine of tyrosine, phenylalanine, valine, methionine and other ninhydrin-positive biological compounds which make difficult the resolution and quantitation of the peaks of interest. On the other hand, the necessity of performing a large number of urine analyses precludes the use of a variety of techniques capable of removing from urine the bulk of interfering compounds[7,10,11] prior to chromatography on the amino acid analyzer. The same may be said about the measurement of total 3-Mehis in urine, as performed by Haverberg *et al.*[13]. This method requires desalting of each hydrolysate on a cation-exchange resin followed by step-wise elution of acidic, neutral and basic amino acids, the latter ones to be concentrated prior to chromatography on the amino acid analyzer.

The fully automated method described here allows in a period of 6 h the direct measurement of free Hyl and its glycosides in whole, filtered urine and of total Hyl and total 3-Mehis in urinary acid hydrolysates. Thus, the method consents the analyses of many consecutive urine samples for the quantitation of products of collagen and muscle proteins metabolism. The measurements of these parameters and especially the measurement of the urinary excretion of 3-Mehis has allowed us to make an

interesting correlation with the loss of weight experienced by American astronauts who spent time in space[14].

EXPERIMENTAL

Preparation of glucosyl–galactosyl hydroxylysine (Glc–Gal–Hyl) and galactosyl hydroxylysine (Gal–Hyl)

These standards were prepared by alkaline hydrolysis of calf collagen type III and IV (Sigma, St. Louis, Mo., U.S.A.) and purified by gel filtration, according to Askenasi's method[6]. The carbohydrate-rich fraction thus obtained was purified further by preparative runs performed with the amino acid analyzer and Askenasi's eluting system[5], run at 69° rather than at 64° (Fig. 1a)). On a repeated run, the effluent was collected in 1 ml fractions and monitored for carbohydrate content with a scaled-down modification of Houle's sulfuric acid reaction[15].

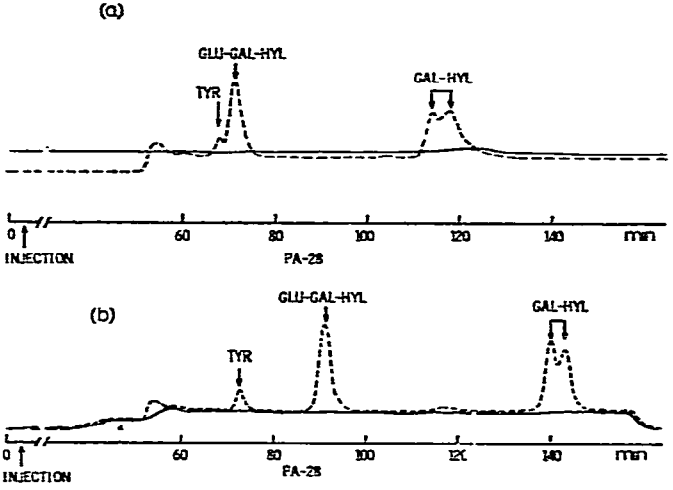

Fig. 1. Chromatogram of a crude preparation of hydroxylysine glycosides from alkali hydrolyzed type IV collagen, performed on 55 × 0.9 cm column of resin PA-28. (a), Askenasi's method, using 0.35 *M* sodium citrate buffer pH 5.28 and a temperature of 69°. (b), three buffer system and temperature program described here.

The central aliquot of the two carbohydrate-containing peaks was collected and run again on the amino acid analyzer with the same method[6]. The first peak (ratio hexoses/Hyl, 2:1) emerged after 72 min. The second one (ratio hexoses/Hyl, 1:1) emerged after 114 and 118 min, being partially resolved in the two diastereoisomers.

An aliquot of Glc–Gal–Hyl was quantitatively converted to Gal–Hyl by hydrolysis with 0.2 *N* HCl for 3 h at 110° These purified preparations of the two glycosides or their crude mixture, as obtained by alkaline hydrolysis of collagen and gel filtration[6] were employed to standardize the new method. The elution profile obtained with our chromatographic system is showr in Fig. 1b.

Preparation of urine samples

Urine was collected during 24-h periods and stored at 4° without preservative.

Aliquots of 3 ml of a 24-h specimen were ultrafiltered through Millipore PSAC membrane (Millipore, Bedford, Mass., U.S.A.), having a nominal molecular weight exclusion limit of 1000.

Aliquots of the ultrafiltrate (0.5 ml) were made alkaline with 50 μl of 2.5 *N* NaOH and ammonia was removed under vacuum. Upon acidification, the whole sample was applied to the long column of the amino acid analyzer (see below) for quantitative measurement of Glc–Gal–Hyl, Gal–Hyl and free Hyl.

Another aliquot of the urine sample (1 ml) was hydrolyzed with 6 *N* HCl for 18 h at 110°. After removal of hydrochloric acid and ammonia under vacuum, the volume of the hydrolysate was readjusted to the original 1.0 ml volume and 0.25 ml was applied to the short column of the amino acid analyzer for the quantitative measurement of total Hyl and total 3-Mehis. Losses of aminoacids due to hydrolysis were accounted for by hydrolyzing under identical conditions an aliquot of amino acid calibration standard type P-B (No. 77729, Hamilton, Reno, Nev., U.S.A.) containing basic amino acids, including 1- and 3-Mehis.

Analysis system

Analyses were performed with a Beckman-Spinco amino acid analyzer (Model 121) equipped with an Infotronics automatic digital-integrator (Model CRS-210) and using a two-column system.

The long column (55 × 0.9 cm), packed with Beckman resin PA-28 (Batch 327659) was eluted with 0.26 *M* sodium citrate, pH = 5.28 (buffer A); 0.35 *M* sodium citrate, pH = 5.26 (buffer B); and 0.20 *M* sodium citrate containing 0.5 *M* NaCl, pH = 6.40 (buffer C). The short one (0.9 × 13 cm), packed with Beckman resin PA-35 (Batch 32435) was eluted with 0.38 *M* sodium citrate, pH = 4.25 (buffer D).

The program has been written so that the two columns are run at the same time. Their respective effluents are collected and mixed with the ninhydrin reagent only at appropriate times (Table I), to avoid precipitation within the reaction coil of the products of reaction between the bulk of acid and neutral aminoacids and the ninhydrin reagent.

TABLE I

COMBINED ELUTION PROGRAM FOR BOTH COLUMNS

Time (min)	*Long column (PA-28)*	*Short column (PA-35)*	*Effluent to reaction coil*	*Temperature (°C)*
0–7	Sample injection, Buffer A	Buffer D	PA-28	69
7–24	A	D	PA-35	69
24–44	A	0.2 *N* NaOH	PA-35	69
44–64	A	D	PA-28	69
64–150	B	Pumping pause	PA-28	69
150–181	C	D	PA-28	Fast cooling system on
181–220	C	Sample injection, D	PA-28	32
220–254	Pumping pause	D	PA-35	32
254–274	0.2 *N* NaOH	D	PA-28	32
274–340	A	D	PA-35	From 32 to 69

For both columns, the flow-rate of the buffers is maintained at 50 ml/h and that of the ninhydrin reagent at 25 ml/h.

The temperature of the columns is kept at 69° for the first 150 min; thereafter, the fast cooling system is switched on and the temperature is lowered to 32° in a period of about 28 min. This change is essential for an optimal resolution of Hyl from both columns. Creatinine was measured on urine ultrafiltrates with Jaffe's reaction[16]. Calculation of the amount of Hyl glycosides was done using the molar ninhydrin color equivalent of Hyl, as suggested by Lon and Hamilton[10].

RESULTS AND DISCUSSION

Elution times and resolution of various compounds of interest (Table II and Fig. 2) are highly reproducible and a complete run lasts about 6 h. The elution of creatinine is very sensitive to pH changes; an increase of 0.02 units in Buffer B causes its elution 8 min earlier, at 147 min, quite close to Gal–Hyl. Therefore, care should be taken to control and readjust the pH at least once a week.

When previously published methods[6–8], designed essentially for the study of collagen hydrolysates, were applied to analyses of urine samples the resolution of Hyl glycosides from neighboring amino acids was not completely satisfactory. On the

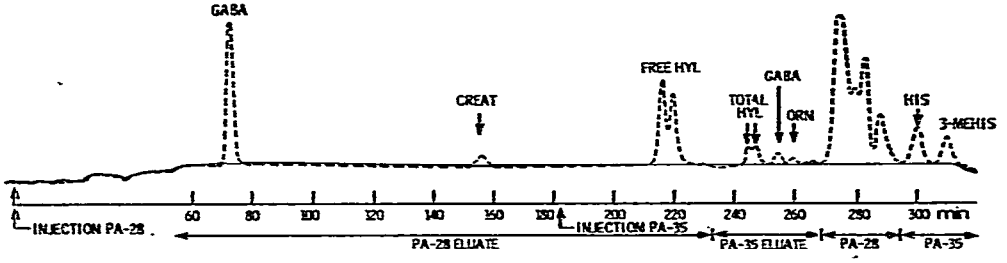

Fig. 2. The elution of various standard amino acids from the two columns, as achieved with the method described. 100 and 30 nmoles of each amino acid were applied to the long and short column, respectively.

TABLE II

ELUTION TIME OF STANDARD COMPOUNDS

Amino acids or ninhydrin positive compounds	*Elution time (min)*	
Tyr	66	PA-28 for urine
γ-Aminobutyric acid (GABA)	74	ultrafiltrate
Phe	76	mol. wt. <1000
Glc–Gal–Hyl	91	
Gal–Hyl	138–143	
Creatinine (Creat)	155	
Glucosamine	187	
Galactosamine	203	
Hyl	212–216	
Hyl	244–246	PA-35 for urine
Histidine	301	hydrolysate
3-Mehis	310	

other hand, the method described by Spiro[9] gave an excellent resolution but required 13 h for each run.

Since we use urine samples not subjected to alkaline hydrolysis prior to analysis, we measure in the effluent of the long column only free Glc–Gal–Hyl, Gal–Hyl and Hyl. Their sum, subtracted from the amount of total Hyl measured with the short column after acid hydrolysis, allows quantitation of peptide-bound Hyl (Fig. 3).

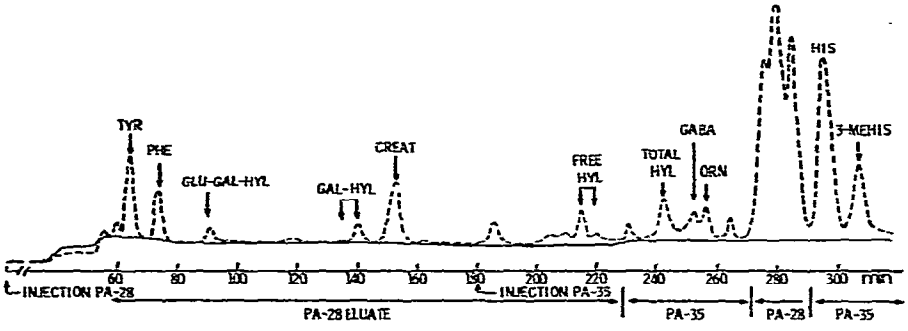

Fig. 3. The pattern obtained with filtered or hydrolyzed normal human urine, using the two columns and the temperature program, as described.

3-Mehis is also measured with the short column on a urine hydrolysate, without need for previous desalting or separation of the basic aminoacids from the acidic and neutral ones. The values obtained represent the sum of 3-Mehis excreted as such and as the N-acetyl derivative[5]. The excretion of the latter represents less than 5% of the total 3-Mehis excreted by human adults and could be disregarded[5]. In the rat, however—and possibly in other animal species—its proportions vary with development and may reach a level u0 to 90% of the total excretion at full maturity[17].

ACKNOWLEDGEMENTS

This investigation was supported in part by a NASA Contract, No. 9-15478; a Research Grant from the Easter Seal Research Foundation of the National Easter Seal Society for Crippled Children and Adults, No. N-7821; and NIH Grant HL-20447-03.

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