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MEASUREMENT OF HYDROXYLYSINE GLYCOSIDES IN URINE AND ITS APPLICATION TO SPINAL CORD INJURY

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SUMMARY

A new technique to evaluate the degradation of skin or bone collagen by measuring glucosylgalactosyl hydroxylysine and galactosyl hydroxylysine is presented. The method utilizes an automated amino acid analyzer. Eluents used are lithium buffers, and the color reagent is ninhydrin. Both glycosides elute in 3.5 h. Samples require minimum preparation. Urinary concentrations of both glycosides in ten patients with cervical spinal cord injuries of less than six months duration were higher than in five healthy controls. Proportional increases were different for each of the two glycosides. Variations in the proportional increase of each glycoside indicate different rates of degradation of skin and bone collagen. Repeated evaluations of the two urinary glycosides may help to predict whether patients are likely to develop skin- or bone-related clinical complications.

INTRODUCTION

Cervical spinal cord injury (SCI) produces an immediate alteration of the collagen metabolism of the affected patients [1]. The specific mechanism whereby the cord injury alters the collagen metabolism is not known. The consequences of bone collagen losses are well documented: greatly increased bone loss leading frequently to hypercalcemia, hypercalciuria, bladder stones, and osteoporosis. In contrast, the consequences of skin collagen loss are not widely documented, although SCI patients present an increased susceptibility to pressure ulcers, suggesting a deterioration of the skin quality. The changes in the collagen metabolism of SCI patients may be monitored by measuring the urinary excretion of collagen metabolites. Two collagen metabolites are especially informative: glucosylgalactosyl hydroxylysine (glu-gal Hyl) and galactosyl hydroxylysine (gal Hyl) (Fig. 1). Glu-gal Hyl is predominant in skin

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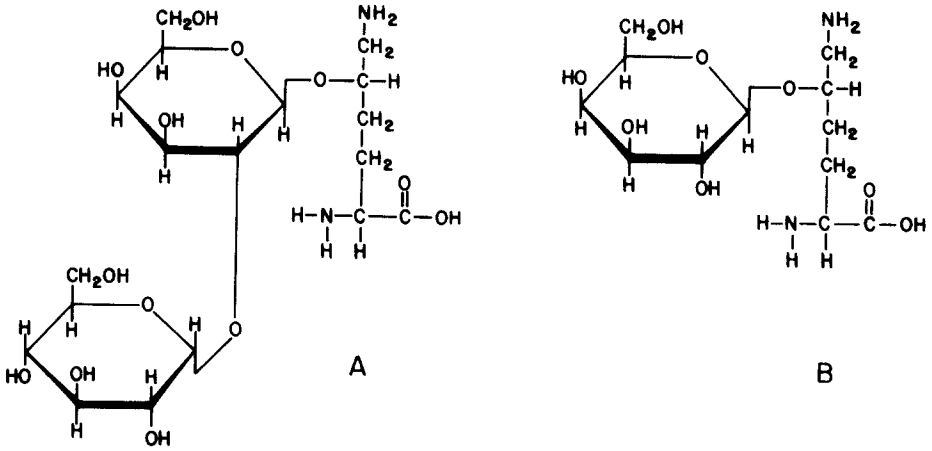

Fig 1 Structural formulae of (A) 2-O-α-D-glucopyranosyl-O-β-D-galactosylpyranosyl hydroxylysine and (B) O-β-D-galactosylpyranosyl hydroxylysine

collagen, and its urinary excretion is increased in patients with skin abnormalities such as erythema multiforme or burns [2—4]. Gal Hyl is predominant in bone collagen, and its urinary excretion is increased in patients with bone disease such as osteomalacia or Paget's disease [2—4]. The relative concentration of the glycosides in the urine of SCI patients would give some indication of the origin of the collagen being degraded and might be predictive of particular complications likely to occur in a specific patient.

The methods previously described to measure urine hydroxylysine glycosides have several disadvantages: (1) multiple columns in the analyzer [5]; (2) incomplete resolution of several amino acids [6]; (3) very lengthy chromatography [7, 8]; (4) mechanical alterations to analyzer [9]; (5) extensive preparation of sample prior to chromatography [4, 10—12].

The method we have developed to measure the urinary concentration of each hydroxylysine glycoside has overcome all these disadvantages. It utilizes an automated single-column amino acid analyzer and completely resolves glu-gal Hyl and gal Hyl in 3.5 h, providing a full amino acid profile in 5 h. It uses a standard commercial amino acid analyzer without any mechanical modification. The alterations to the elution buffer system which make the separation possible are straightforward and preparation of the sample prior to chromatography is minimal.

EXPERIMENTAL

Glu-gal Hyl and gal Hyl are not available commercially. They were extracted from the anterior lens capsule of dog lenses by a modification of Askenasi's method [6]. The lens capsules were homogenized in 0.15 *M* sodium chloride and lyophilized. Portions of the dried homogenate were hydrolyzed in 2 *M* lithium hydroxide at 110°C for 18 h. The hydrolysate was adjusted to pH 6 with perchloric acid and centrifuged at 60,000 *g* for 15 min at 4°C. Portions of the supernatant were placed on a 124 × 0.9 cm column filled with Bio-Gel P-2, 400 mesh, resin and eluted with 0.1 *M* pyridine—acetate buffer, pH 4.8.

Fractions of 3.7 ml were collected (LKB Model 2070 fraction collector) and tested for the presence of hexoses by the method of Dubois et al. [13], and for the presence of amino acids by the method of Moore and Stein [14]. Fractions positive for both were subjected to acid hydrolysis in 2 *M* hydrochloric acid for 3 h at 109°C; an increase in free Hyl content is an indication of the presence of either or both glycosides.

The amino acid analyzer used was Beckman's Model 119CL provided with a data processor Model 126 capable of integrating the peaks in the chromatogram produced by the attached Bristol recorder. The analyzer column was 460 × 6 mm, filled with a cross-linked sulfonated sytrene copolymer resin (Beckman W3P) to a height of 22 cm. The resin was changed to the lithium form by treatment with lithium hydroxide. The eluting buffers were made from Beckman concentrates diluted according to directions but altered in the following way: (a) Beckman 0.2 *M* lithium citrate buffer, pH 2.83 was changed to pH 2.70 (buffer A); (b) to Beckman 0.2 *M* lithium citrate buffer, pH 3.70, 2 g of lithium chloride per l of buffer was added and pH changed to 3.21 (buffer B); (c) to Beckman 1 *M* lithium citrate buffer, pH 3.75, 6% 2-propanol was added and pH changed to 3.68 (buffer C). The color reagent was a standard ninhydrin solution using stannous chloride as a reducing agent. Starting temperature was 40°C, raised to 65°C after 40 min. Buffer flow-rate was 44 ml/h. Ninhydrin flow-rate was 22 ml/h. Buffer A ran for 66 min, buffer B for 101 min, and buffer C for 98 min (Table I).

TABLE I

ELUTION PROGRAM FOR THE AMINO ACID ANALYZER

Buffer (lithium)	Additive	pH (25°C)	Pumping time (min)	Temperature
A 0.2 *M*	—	2.70	66	40°C for 40 min then 65°C
B 0.2 *M*	2 g lithium chloride per l	3.21	101	65°C
C 1.0 *M*	6% 2-propanol	3.68	98	65°C

Urine samples from five healthy controls and ten spinal cord injury patients were assayed for their glu-gal Hyl and gal Hyl concentrations using the present method. The controls were males between 30 and 52 years of age. The patients were males, injured less than six months, between 14 and 50 years of age, with no previous history of chronic diseases. Both controls and patients gave informed consent to participate in the project and provided 24-h urine samples. The urines were kept cold during the collections and frozen if not assayed immediately. Ammonia was removed from the sample aliquots by raising the pH between 11.5 and 12.0 with 4 *M* lithium hydroxide and placing them in a dessicator containing concentrated sulfuric acid under reduced pressure for 6 h. The pH of the urine was then adjusted to pH 2.2 with 6 *M* hydrochloric acid,

filtered through a Millipore 0.22-μm filter and brought back up to its original volume with 0.2 *M* lithium citrate buffer, pH 2.2. An aliquot of 100 μl was applied to the amino analyzer column. In accordance with the findings of Lou and Hamilton in 1971 [7], the molar ninhydrin color equivalent for Hyl was used to calculate the glycoside concentrations. Results of the assay of the control and patient urine samples were compared by analysis of variance by means of a taped program of the Wang table top computer.

RESULTS

Fractions 15 through 24 of the Bio-Gel P2 eluate of the lens hydrolysate tested positive for both hexoses and amino acid (Fig. 2). When each of these fractions was subjected to acid hydrolysis, only fraction 15 exhibited an increase in free Hyl content (Figs. 3 and 4). This fraction was used to adjust the elution protocol of the amino acid analyzer to elicit the desired resolution of glu-gal Hyl and gal Hyl in the chromatogram. Since only 5% of the glycosylated Hyl in lens capusule is the monosaccharide [15], fraction 15 was subjected to a mild acid hydrolysis in 0.2 *M* hydrochloric acid for 4 h at 109° C. This releases only the terminal glucose residue in some of the molecules of glu-gal Hyl and makes it possible for the analyzer to detect the augmented gal Hyl [6, 7, 9, 16]. With the present protocol, the retention time for glu-gal Hyl was approximately 115 min and for gal Hyl 185 min (Fig. 5).

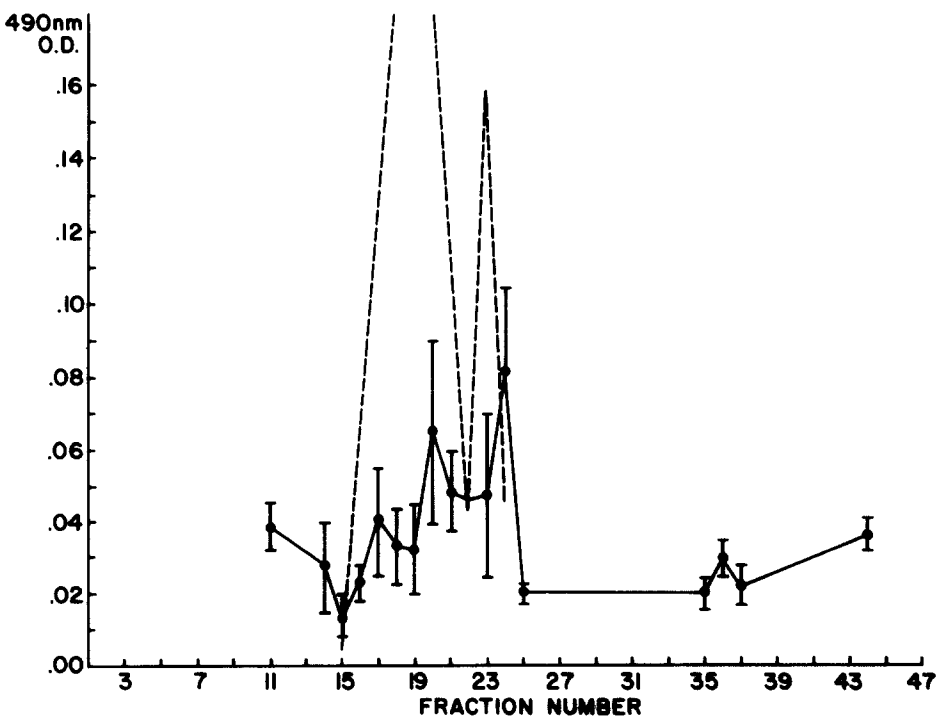

Fig. 2. Hexose determinations in Bio-Gel P2 filtrate fractions. Average of determinations in four separate filtrations. Bars indicate maximum variation. Abscissa: fractions numbers; ordinate: optical density at 490-nm wavelength; (- - -), largest amino acid peaks

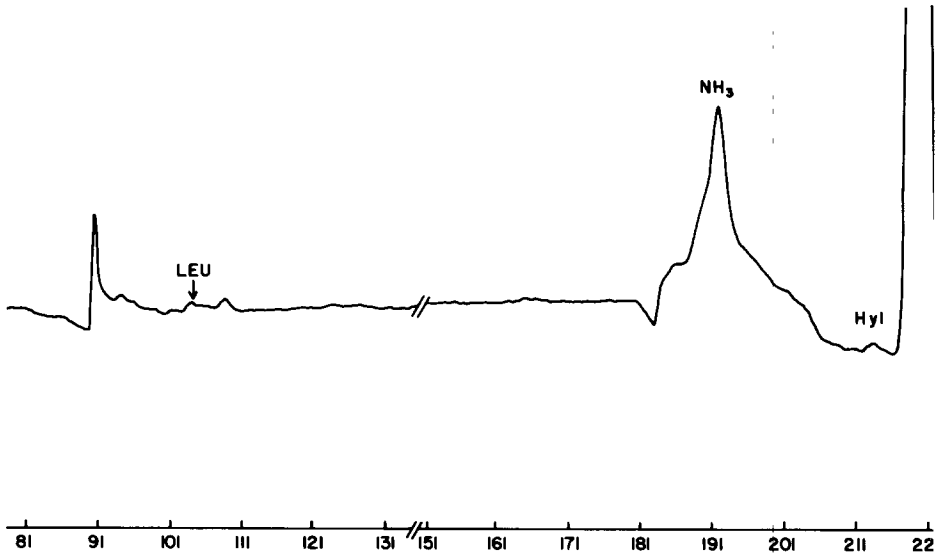

Fig. 3. Amino acid analyzer chromatogram of Bio-Gel P2 filtrate fraction No. 15. Curve represents the sum of the absorptions at 440 and 570 nm. Only sections of interest of the chromatogram are shown. Abscissa: retention times in min; ordinate: absolute units proportional to optical density. Size of sample: 100 μl; recorder set at highest sensitivity (0.1 absorption).

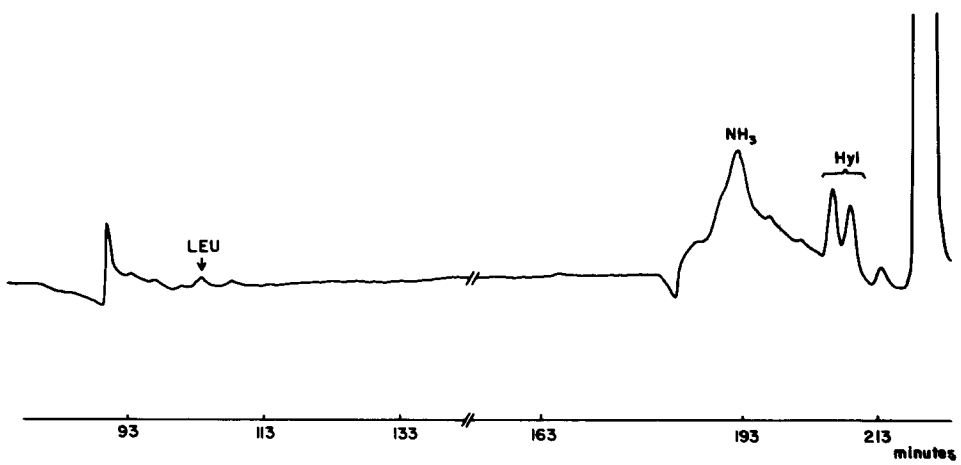

Fig. 4. Amino acid analyzer chromatogram of the same fraction as in Fig. 3 after it had been subjected to acid hydrolysis in 2 *M* hydrochloric acid for 4 h at 109°C and brought to pH 3 with 4 *M* lithium hydroxide. Curve represents the sum of the absorptions at 440 and 570 nm. Only sections of interest of the chromatogram are shown. Abscissa: retention times in min; ordinate: absolute units proportional to optical density. Size of sample: 200 μl; recorder set at highest sensitivity.

The 24-h urine samples from five healthy controls and ten spinal cord injury patients were assayed using the protocol outlined in Table I. The mean concentration of glu-gal Hyl in the healthy controls was 18.7 ± 2.4 (S.E.M.) μmol per g creatinine. The mean gal Hyl in the healthy controls was 12.2 ± 3.8 (S.E.M.) μmol per g creatinine. In spite of the large differences between the means of

the two groups [31.8 (S.E.M.) μmol per g creatine for glu-gal Hyl and 20.5 (S.E.M.) μmol per g creatinine for gal Hyl], these differences were not statistically significant unless we discard the results on patient 9 (Table II). This patient's hydroxyproline was also extremely high, 266 mg per g creatinine.

Fig. 5. Amino acid analyzer chromatogram of the same sample as in Fig. 3 but done under the final parameters chosen for this method. Only the section of interest of the chromatogram is shown. Curve represents the sum of the absorptions at 440 and 570 nm. Abscissa: retention times in min; ordinate: absolute units proportional to optical density; size of sample: 625 μl; recorder set at highest sensitivity (0.1 absorption).

TABLE II

CONCENTRATIONS OF GALACTOSYL HYDROXYLYSINE AND GLUCOSYLGALACTOSYL HYDROXYLYSINE IN CONTROLS AND PATIENTS

Sample	Controls			Patients		
	glu-gal (μmol per g creatinine)	gal (μmol per g creatinine)	glu-gal/gal	glu-gal (μmol per g creatinine)	gal (μmol per g creatinine)	glu-gal/gal
1	16 7	9.6	1.7	20.4	15.5	1.3
2	13.9	9.3	1.5	44.5	30.2	1.5
3	21.2	14.0	1.5	30.0	14 9	2.0
4	26.8	18.2	1.5	42.9	23.5	1.8
5	14.7	10.1	1 5	84.3	25 4	3.3
6				27.0	24.8	1.1
7				35.2	24.6	1.4
8				28.5	21.8	1.3
9				142.7	106.0	1.3
10				49.7	40.3	1.2
Mean	18.7	12.2		50.5	32 7	
S.D.	5.4	3.8		37.1	26.7	
S.E.	2 4	1.7		11.8	8.5	
p				>0.05	>0 1	
If sample 9 is eliminated.			Mean	40.3	24.6	
			S.D.	19.0	7.6	
			S.E.	6.3	2.5	
			p	0.05	0.01	

To analyze the inter-assay variation, a random urine sample was analyzed eight times. The determinations were carried out over a period of three weeks, utilizing several different batches of the eluting buffers and color reagent. The mean of the eight glu-gal Hyl determinations was 17.2 ± 0.2 (S.E.M.) μmol per g creatinine.

To analyze the intra-assay variation, a different random urine sample was analyzed ten consecutive times. The determinations were carried out over a period of three days, utilizing the same batches of eluting buffers and color reagent. The mean of the ten glu-gal Hyl determinations was 11.0 ± 0.3 (S.E.M.) μmol per g creatinine.

DISCUSSION

The method described herein is a straightforward procedure adaptable to many uses. It has five main positive features: (1) the chromatography is carried out in a single-column Beckman 119CL amino acid analyzer with the resin in the lithium form, and only three buffers are needed; (2) in the urine chromatograms, glu-gal Hyl and gal Hyl peaks are clearly resolved from the other peaks and accurately read and quantitated by the data analyzer; the magnitude of the inter- and intra-assay variations attest to the reproducibility of the method; (3) only 100 μl of urine are required for the chromatography; (4) the preparation of the urine sample requires little handling or chemical alteration; (5) both glycosides elute within 3.5 h.

The concentration of glu-gal Hyl and gal Hyl found in the control group had a narrow range of values which are similar to those obtained by previous authors (Table III). In contrast, the concentration of the glucosides in the patient group exhibited a very wide range of values. The mean values in both groups were very different, but, because of the large variation in the patient group, these differences were not statistically significant unless we disregard the results for patient 9. Nevertheless, a trend can be seen (Fig. 6). Of the patients 90% had glu-gal Hyl values above the control mean and all patients had gal Hyl values above the control mean. The glu-gal Hyl/gal Hyl ratio in the patient

TABLE III

COMPARISON OF NORMAL VALUES IN URINE FOR HYDROXYLYSINE GLYCOSIDES CITED IN THE LITERATURE WITH THOSE OBTAINED BY PRESENT METHOD

glu-gal Hyl (μmol per g creatinine)	gal Hyl (μmol per g creatinine)	glu-gal Hyl (μmol per 24 h)	gal Hyl (μmol per 24 h)	glu-gal Hyl/ gal Hyl	Reference
16.5	11.0	26.?	17.7	1.5	3
17.8	11.6	21.5	13.7	1.6	18
—	—	31.9	28.3	1.1	11
20.0	12.0	—	—	1.7	10
30.0	20.0	—	—	1.6	19
14.0	8.0	—	—	1.7	15
18.7	12.0	28.9	18.7	1.5	present paper

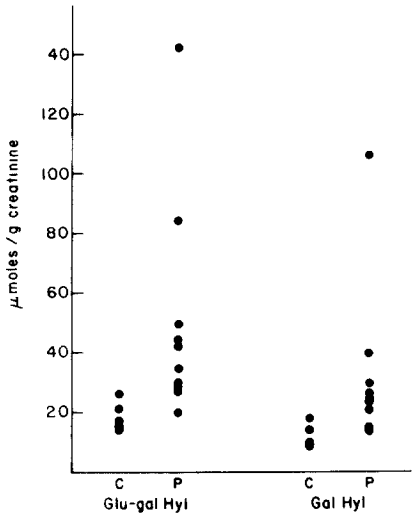

Fig. 6. Distribution of values of hydroxylysine glycosides in the urine of controls (C) and patients (P).

group had a wide range of values. A longitudinal study in a large population will be needed to determine whether a high ratio will increase the probability that the patient will develop skin complications and a low ratio will be associated with bone-related complications.

This method was developed to explore the metabolism of collagen in spinal cord injury. Previous data indicated that trauma of the spinal cord causes an increased bone mineral and collagen metabolism. The collagen loss occurs first in all the bones, later only in the bones of the paralyzed body areas [1]. In addition, there is a loss of collagen from the skin [17]. The urinary concentrations of glu-gal Hyl and gal Hyl will be an indication not only of total collagen loss after spinal cord injury, but also of which tissue is most at risk.

The content of hydroxylysine varies with the type of collagen. In human skin collagen, glu-gal Hyl represents 61% of the total glycosides, while in bone collagen it represents only 13—40% [4, 18]. The relative and absolute concentrations of the hydroxylysine glycosides excreted in urine indicate the tissue origin of the collagen metabolites and the rate of the degradation of the collagen [3, 11, 15, 19]. Measuring the excretion of these glycosides could be a means of monitoring the effect of the therapies used to minimize the sequelae of spinal cord injury (osteoporosis, skin ulcers, etc.), as well as evaluating the progress and the efficacy of the therapy in many diseases that affect collagen (e.g., Ehler-Danlos syndrome, hyperthyroidism, chronic uremia).

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