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CHROMATOGRAPHIC ANALYSIS OF HYDROXYLYSINE GLYCOSIDES AND ACID HYDROLYZATES

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SUMMARY

A single-column chromatographic technique for the analysis of hydroxylysine glycosides and acid hydrolyzates is described. This technique employs a Durrum D-500 amino acid analyzer equipped with a standard 48 cm × 1.8 mm column packed with DC-4A resin. Resolution was achieved with four sodium citrate buffers and four column temperatures. Products of glycoprotein hydrolysis including cysteic acid, methionine sulfoxide, 4-hydroxyproline, α-aminobutyric acid, glucosamine, galactosamine, hydroxylysine, tryptophan and the internal standard, norleucine, are resolved. The completely automated procedure takes 185 min per run and can measure amino acid residues in the nanomole range.

INTRODUCTION

The quantitation of hydroxylysine glycosides is important in studies of the nature of collagens and basement membranes. Hydroxylysine glycosides are involved in cross-linking of collagen chains[1]. It has been proposed that elevations in hydroxylysine glycoside content of glomerular basement membrane in the diabetic state alter the packing and molecular organization of the membrane's component peptide chains[2]. Chromatographic techniques for quantitation of hydroxylysine glycosides exist[3–8] but have certain limitations: analysis times are long[3,4], resolution of hydroxylysine glycosides from other amino acid residues is poor[3], preliminary removal of interfering amino acids is required[3,5] and capability to resolve products of acid hydrolysis of glycoproteins is limited[5–8]. Georgiadis *et al.*[9] have described an automated procedure that uses a Durrum D-500 amino acid analyzer for single-column analysis of acid hydrolyzates. The present communication describes a modification of that procedure that allows analysis of hydroxylysine glycosides as well as of the products of acid hydrolysis. Complete analysis takes 185 min. Reproducibility in the 10^{-9} mole range is within 5% of the mean value.

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EXPERIMENTAL

Collagen hydrolyzate standards (Lot No. 04075 11) and Nin-Sol, a premixed solution of ninhydrin in dimethylsulfoxide, were purchased from Pierce (Rockford, Ill., U.S.A.). Elution buffers were prepared from reagent-grade citric acid from Mallinckrodt (St. Louis, Mo., U.S.A.) and from "Baker Analyzed" sodium citrate from J. T. Baker (Clifton, N.J., U.S.A.). All other chemicals used were reagent-grade or better. Bovine anterior lens capsules were isolated from cow eyes according to the procedure of Fukushi and Spiro[10].

Preparation of hydroxylysine-linked carbohydrate units

Glucosylgalactosylhydroxylysine (GGH) was prepared from bovine anterior lens capsules by gel-permeation and ion-exchange chromatography using a variation of the procedures of Cunningham *et al.*[11] and of Smith[12]. Lens capsules (40 mg) were hydrolyzed in 2.5 ml of 2 *N* NaOH in a sealed polypropylene tube at 105° for 22 h. The hydrolysate was neutralized with HCl and lyophilized. The residue was dissolved in 0.05 *M* pyridine acetate, pH 4.8, and chromatographed in the same buffer on a 140 × 1.5 cm Bio-Gel P-2 column (200–400 mesh) at a flow-rate of 0.5 ml/min. Anthrone-positive material[13] was pooled, lyophilized, redissolved in water and chromatographed again on a 22 × 1.5 cm column of Bio-Rad AG 50W-X2 (200–400 mesh) cation-exchange resin with a linear gradient of HOH against 3 *M* NH_4OH at a flow-rate of 1.7 ml/min. Anthrone-positive material was again pooled, lyophilized and rechromatographed on the AG 50W-X2 column in step gradients of water, 0.2 *N* sodium citrate at pH 3.25 and 0.2 *N* sodium citrate at pH 4.2, followed by a continuous gradient of 0.2 *N* sodium citrate at pH 4.2 against the same sodium citrate buffer with 0.2 *N* NaCl added. Carbohydrate content of anthrone-positive peaks was analyzed in a Hewlett-Packard 5831 gas–liquid chromatograph according to the procedure of Clamp *et al.*[14]. The final chromatographic step yielded a peak with a glucose/galactose/hydroxylysine ratio of 1.0:0.99:1.07. This fraction was desalted on the Bio-Gel P-2 column and subsequently used as the stock solution.

Galactosylhydroxylysine (GH) was prepared from Rock Island deep sea sponge[15] by an extension of the procedure described above. After alkaline hydrolysis and neutralization, the sample was centrifuged at 18,000 *g* for 20 min; the supernatant was lyophilized and desalted on the Bio-Gel P-2 column. The anthrone-positive material was dissolved in water and concentrated H_2SO_4 was added to bring the solution to pH 2; then it was hydrolyzed for 22 h at 100°. Chromatography on the AG 50W-X2 column with the step gradients described previously yielded a peak that had a galactose/hydroxylysine ratio of 1.0:1.06. This fraction was desalted on the Bio-Gel P-2 column and used as a stock solution of GH.

Preparation of samples for analysis

Bovine lens capsules were lyophilized extensively just prior to use. Norleucine was added and the lyophilized mixture was hydrolyzed for 24 h at 105° in 2 *N* NaOH (1 ml/10 mg capsule) in stoppered polypropylene tubes that were sealed in test tubes containing a few milliliters of water. The hydrolyzate was neutralized on a column of Bio-Rad AG 50W-X2 (200–400 mesh), hydrogen form, with an exchange capacity at least 5 times that of the charged components in the hydrolyzate. The column was then

washed with 10 bed volumes of water, eluted with 10 bed volumes of 1.5 *M* NH_4OH, and lyophilized. The residue was resuspended in water prior to loading on the amino acid analyzer.

Acid hydrolysis was performed using 3 *N* *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (TSA reagent) according to the procedure of Liu and Chang[16]. A 1-ml volume of TSA reagent was added to every 5.6 mg of lens capsule.

Analysis system

We analyzed the samples with a Durrum D-500 amino acid analyzer (Durrum, Sunnyvale, Calif., U.S.A.), which utilizes a standard 48 cm × 1.8 mm column prepacked with DC-4A resin (8 ± 2 μm diameter particles). Two pens recorded absorbance at 590 and 440 nm simultaneously. With a pump stroke setting of 0.075 in., buffer flow-rate was 8.6 ml/h. Pressures generated ranged from 2150 p.s.i. at the beginning of the run to 1450 p.s.i. when the last buffer was being pumped. The details of the elution system are shown in Table I.

TABLE I

ELUTION SEQUENCE

pH was adjusted with HCl. All pH measurements were at room temperature (23°). All citrate buffers contained 1 ml pentachlorophenol (5 mg/ml in 95% ethanol) per l. Initial temperature was 48°; it was raised to 54°, 65° and 70° at 44.52, 83.00 and 147.00 min, respectively. Sample was loaded at 35 min for a duration of 5 min.

Time (min)	*Eluent*
0.83– 11.08	0.3 *M* NaOH (for column flushing)
11.00– 46.47	0.18 *M* Na^+, 0.067 *M* citrate, 0.5% thiodiglycol, 10% methanol, pH 2.90 ± 0.01
44.52–124.73	0.18 *M* Na^+, 0.067 *M* citrate, 0.5% thiodiglycol, pH 3.27 ± 0.01
123.50–143.20	0.18 *M* Na^+, 0.067 *M* citrate, 0.5% thiodiglycol, pH 4.55 ± 0.01
143.22–185.00	1.0 *M* Na^+, 0.133 *M* citrate, pH 7.00 ± 0.01

The prime advantage of a multibuffer system is the flexibility it provides in positioning components in a given region without compromising resolution elsewhere in the chromatogram. The principal differences of our procedures from that of Georgiadis *et al.*[9] are as follows. The methanol content in the pH 2.90 buffer was increased to improve resolution in the initial portion of the elution profile, elution time with pH 3.27 buffer was extended to make room for GGH, and column temperature and pH of the last buffer were both raised to speed up elution of the basic amino acids.

RESULTS AND DISCUSSION

Purified GGH and GH

Chromatography of our preparations of GGH and GH indicated that both preparations were virtually free of other amino acids. Hydrolysis in TSA reagent and analysis for hydroxylysine, with norleucine as an internal standard, yielded a GGH/OHLys ratio of 1.25. Comparison with the Pierce standard solution yielded a GGH/

Leu ratio of 1.27. GH/OHLys and GH/Leu ratios are 1.13 and 1.15, respectively. The reason for the discrepancies from previously published ratios[4,8] is not clear.

Standard mixtures

Fig. 1 shows the elution pattern of a mixture of amino acids with GGH and GH. The elution pattern is identical to that obtained by Georgiadis *et al.*[9] with the following exceptions. The relative positions of valine and cystine and of lysine and ammonia are reversed, and glucosamine and galactosamine are eluted relatively earlier in our system. There is baseline separation of most of the peaks. The only major overlap is that of GGH and galactosamine. However, since the sugars of GGH are cleaved by acid hydrolysis and galactosamine is totally destroyed by base hydrolysis[17], the two compounds cannot coexist in the type of acid–base hydrolyzates used for these analyses.

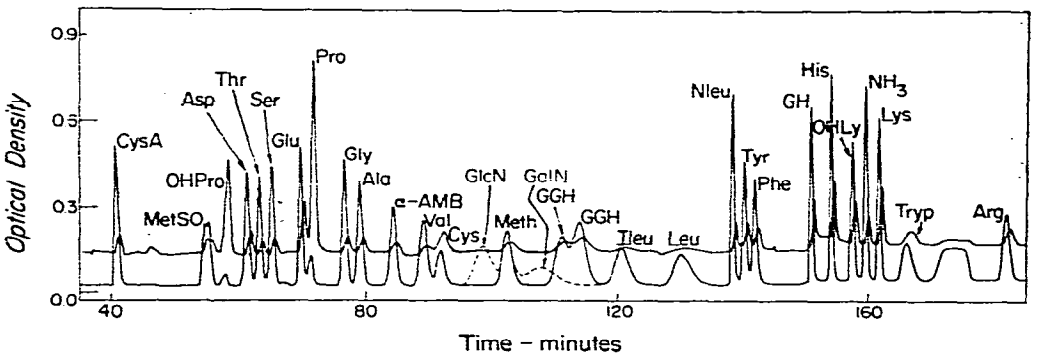

Fig. 1. Chromatogram of a standard mixture of amino acids, containing 17.6 nmoles GGH, 6.7 nmoles GH, 4.25 nmoles cystine, 42.5 nmoles OHPro, 42.5 nmoles Pro, 12 nmoles GlcN and GalN and 8.5 nmoles of each of the other amino acids. α-AMB is α-aminobutyric acid. Upper tracing, absorbance at 440 nm; lower tracing, absorbance at 590 nm. The broad peak after tryptophan is an artifact of the elution buffer

The elution times of the amino acids that elute near the amino sugars are sensitive to the pH of the elution buffer and to column operating pressure. In contrast, the elution times of the amino sugars are less sensitive to pH and to operating pressure. Hence, by varying these parameters it is possible to position the amino sugars between the amino acids for maximum resolution. The resolution of glucosamine and galactosamine in our system is comparable to that obtained in previously published procedures[5,7,9,17–19].

The chromatograms shown in Figs. 1 and 2 were run at photometer settings of 1.0 at both 590 and 440 nm. By changing the settings to 0.1, the most sensitive photometer setting, we can analyze nanomole quantities of the individual amino acids. The resolution remains good, but errors increase because of increased baseline variation. In 10 runs of a standard mixture containing 2 nmoles of each amino acid (except proline and hydroxyproline, 10 nmoles) in each run, the average standard deviation of the ratio of each amino acid to norleucine was less than 5% of the mean value.

Stability of residues during base hydrolysis

Hydrolysis of GGH in 2 *N* NaOH (200 nmoles/ml) at 105° for 24 h resulted in a 4.6% conversion to both GH and hydroxylysine, with 90.8% remaining un-

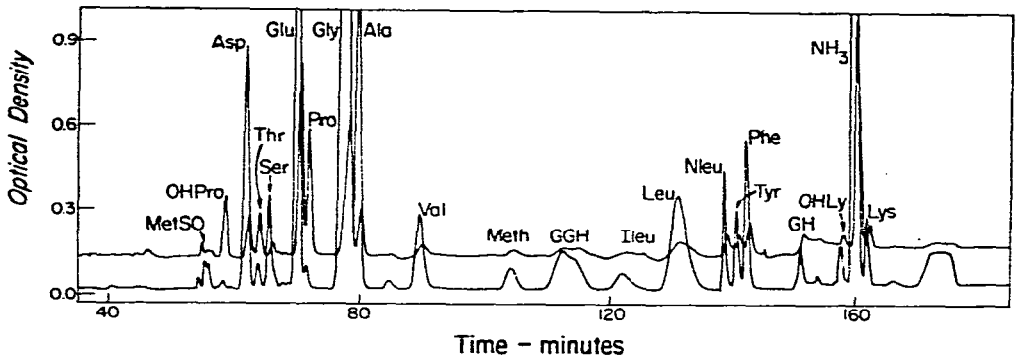

Fig. 2. Chromatogram of base-hydrolyzed bovine anterior lens capsule (56 μg) and 5.6 nmoles of norleucine. Upper tracing, absorbance at 440 nm; lower tracing, absorbance at 590 nm.

changed. Similar base hydrolysis of GH resulted in conversion of 10% of this glycoside to hydroxylysine. These degradative losses are comparable to the 15% losses observed previously under similar hydrolysis conditions[20].

The ratios of the amino acids to norleucine changed substantially after hydrolysis in 2 *N* NaOH. When the standard mixture (Fig. 1), with 13 nmoles of norleucine, was hydrolyzed in 0.05 ml of 2 *N* NaOH for 24 h at 105°, only leucine, isoleucine, hydroxylysine and glutamic acid had amino acid/norleucine ratios within 6% of those obtained after hydrolysis in TSA reagent. Comparison of the hydrolysis products of cow anterior lens capsule, after 24-h base hydrolysis and after hydrolysis in TSA reagent, showed that only leucine values are the same after acid and after base hydrolysis. This finding suggests that leucine alone is an acceptable bridge between analyses of TSA and base hydrolyzates. This is in agreement with the data of Spiro[20] on kidney glomerular basement membrane. Although it is possible to estimate hydroxylysine glycosides using leucine as a bridge, errors could occur if hydrolytic cleavage by alkali is incomplete, causing the extent of release of leucine and hydroxylysine glycosides to vary. In assays of amino sugars, which are released under relatively mild hydrolytic conditions, no constituent amino acid can serve as a satisfactory bridge. More reliable quantitative data can be obtained if a stable internal standard is added to samples prior to hydrolysis.

Norleucine is ideal for use as an internal standard. It is stable to both acid and base hydrolysis. After 24-h base hydrolysis, less than 1% of norleucine was lost and after 22-h TSA hydrolysis, less than 1.5% was lost (for these stability experiments other amino acids were added as standards after hydrolysis).

The most accurate method for the estimation of GGH and GH, therefore, involves analysis of samples to which known amounts of norleucine have been added, and which are hydrolyzed for different durations. This verifies the completeness of GGH and GH release. Subsequent corrections for degradative losses sustained during base hydrolysis yield estimates of the absolute quantities of these hydroxylysine glycosides.

Analysis of cow anterior lens capsule

The GGH, GH and amino acid content of cow anterior lens capsule were determined. We analyzed samples obtained after hydrolysis in TSA reagent and in NaOH for 24, 48 and 72 h. Hydrolysis in NaOH for 24 h released 96.5% of hydroxy-

lysine and its glycosides. A representative chromatogram of a 24-h base hydrolyzate is shown in Fig. 2. The composition we obtained was in close agreement with those obtained by Spiro and Fukushi[10,21] and by Kefalides[22]. The most noteworthy differences were in the relative amounts of hydroxylysine, GGH and GH. We found 37.6, 30.2 and 3.4 residues/1000 amino acid residues, respectively, whereas Spiro and Fukushi obtained 44.8, 35.25 and 2.25 residues/1000, respectively. However, although the total amount of the various forms of hydroxylysine differs, the extent of glycosylation was found to be essentially the same (Spiro and Fukushi, 84%; our work, 89%).

Advantages of this technique

The single-column technique described in this paper offers several advantages. It is an integrated scheme for analysis of the products of different types of hydrolysis. Thus, GGH, GH, amino acids, glucosamine and galactosamine can all be analyzed in the same chromatographic system and their relative proportions calculated on the basis of the common internal standard, norleucine. This offers the advantage of procedural simplification and eliminates errors produced by the use of different techniques to analyze different components. The system offers high sensitivity (nanomole range) coupled with good reproducibility. Thus, the amino acids, glucosamine, galactosamine, GGH and GH in 20 to 100 μg of glycoprotein can be estimated. Only a short time (185 min) is required for each chromatographic run. Since sample loading on the Durrum D-500 is automated, up to eight analyses can be completed daily.

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