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A RAPID AUTOMATIC ANALYSIS OF HYDROXYLYSINE, HYDROXYLYSINE GLYCOSIDES, AND METHIONINE SULFOXIDE IN COLLAGEN AND BASEMENT MEMBRANE*,**

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SUMMARY

A procedure has been developed which allows the simultaneous determination of methionine sulfoxide, hydroxylysine, and hydroxylysine glycosides in alkaline hydrolysates of collagen and basement membrane. This procedure involves the separation of the above components from the remaining amino acids on an amino acid analyzer using a single-column of Beckman type AA-15 resin. Complete resolution was achieved with a stepwise four-buffer elution program in 3.5 h. The analysis is completely automated with a Bio-Cal AS-20 multi-sample programmer, which automates stepwise buffer changes, temperature changes, regeneration, equilibration, sample injection, and final shutdown after a programmed series of up to nineteen consecutive samples.

INTRODUCTION

Collagen and basement membrane, a collagen-related protein, contain a portion of their hydroxylysine residues glycosidically linked to monosaccharide and disaccharide units, O-β-D-galactosylhydroxylysine (gal-hyl) and 2-O-α-D-glucosyl-galactosylhydroxylysine (glc-gal-hyl), respectively[1]. Several methods have been developed for the quantitative estimation of these carbohydrate units as well as for unsubstituted hydroxylysine in alkaline hydrolysates of these proteins. The earlier method developed by Spiro[1] required the use of several chromatographic steps, which included gel filtration, paper, and ion-exchange chromatography, and finally analysis on an amino acid analyzer in order to resolve these components from the remaining amino acids in the alkaline hydrolysates. This method was modified by Pinnell *et al.*[2] for direct determination of the hydroxylysine glycosides and

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unsubstituted hydroxylysine on an amino acid analyzer after prior removal of the bulk of the amino acids in the hydrolysate by gel filtration chromatography; however, resolution of components was incomplete. Recently Spiro[3] developed a direct analysis procedure employing a single-column system on an amino acid analyzer which requires an 11.5-h completion time. A similar procedure was developed by Kimura[4], using an unspecified ion-exchange resin and requiring a 5.5-h completion time.

This paper describes a fully automated amino acid analyzer procedure requiring 3.5 h for the direct analysis of unsubstituted hydroxylysine and hydroxylysine glycosides. Moreover, this procedure allows the simultaneous determination of methionine sulfoxide, a constituent of basement membranes which previously required a separate analysis for its estimation in alkaline hydrolysates[5].

EXPERIMENTAL

Preparation of glomerular basement membrane

Glomerular basement membrane was prepared from bovine glomeruli as described by Spiro[6].

Preparation of hydroxylysine-linked carbohydrate units

Glc-gal-hyl was isolated from alkaline hydrolysates of bovine glomerular basement membrane essentially as described by Spiro[7] and further purified by ion-exchange chromatography. Basement membrane (80 mg) was hydrolyzed in 5 ml of 2 *N* NaOH in sealed polypropylene tubes at 100° for 24 h. The hydrolysate was neutralized with 6 *N* HCl and lyophilized. The residue was dissolved in 2.0 ml of 0.1 *M* pyridine acetate buffer, pH 4.8, and applied to a column (1.2 × 175 cm) packed to a height of 165 cm with Bio-Gel P-2 (200–400 mesh) and equilibrated with the same buffer. Elution was achieved with the pyridine acetate buffer at a rate of 15 ml/h and 2-ml fractions were collected. Aliquots of these fractions were analyzed for hexose by the anthrone reaction[8] and for amino acids by the ninhydrin reaction[9]. The elution profile for this column is shown in Fig. 1. The peak containing the carbohydrate (glc-gal-hyl) was pooled and lyophilized. A stock solution of glc-gal-hyl of approximately 0.9 μmole/ml was prepared and used as a standard in the initial studies to establish an elution program for separating this substance from standard amino acids on the analyzer. Gal-hyl was prepared for the initial studies by hydrolyzing an aliquot of the glc-gal-hyl stock solution with 0.1 *N* HCl for 9 h in a sealed tube.

Glc-gal-hyl and gal-hyl were further purified for use in determining color factors by ion-exchange chromatography utilizing the amino acid analyzer procedure described below for the separation of these components from amino acids. An aliquot of the fraction (10 μmoles) from the Bio-Gel P-2 column was applied to the amino acid analyzer column and eluted with the four-buffer stepwise system. The column effluent line was disconnected from the ninhydrin bath and fractions collected at 1-min intervals. Aliquots of the fraction were analyzed for hexose by the anthrone reaction[8]. Fractions containing glc-gal-hyl were pooled, lyophilized, and desalted on the Bio-Gel P-2 column described in Fig. 1. The carbohydrate peak was well separated from the buffer salts as monitored by the anthrone reaction[8] and

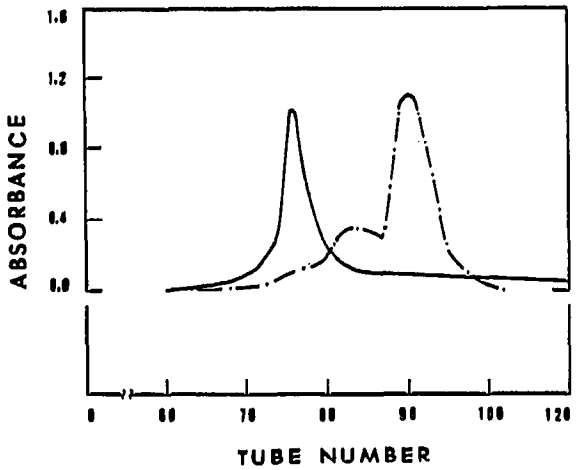

Fig. 1. Gel filtration on Bio-Gel P-2, 200–400 mesh, of alkaline hydrolysate of bovine glomerular basement membrane (80 mg). The solid line shows hexose by the anthrone reaction and the broken line shows amino acid by the ninhydrin reaction.

conductivity measurements. Gal-hyl (5 μmoles) was purified in a similar manner after conversion of the purified glc-gal-hyl to gal-hyl by hydrolysis with 0.1 *N* HCl at 100° for 9 h in a sealed tube.

Preparation of samples for analysis

Glycoprotein samples, basement membrane, calf-skin collagen (Calbiochem, Los Angeles, Calif., U.S.A.), and thyroglobulin (Nutritional Biochemicals, Cleveland, Ohio, U.S.A.), were hydrolyzed with 2 *N* NaOH at a concentration of 15 mg/ml for 24 h at 100°. After hydrolysis samples were neutralized with an equivalent amount of HCl and stored frozen until analyzed. Samples which were analyzed for methionine sulfoxide, in addition to glc-gal-hyl, gal-hyl and hydroxylysine, were hydrolyzed with alkali as described above except tubes were sealed under nitrogen as previously described[5].

Analysis system

Quantitative analysis of glc-gal-hyl, gal-hyl, hydroxylysine, and methionine sulfoxide were performed on a Beckman-Spinco Model 120 C amino acid analyzer (Beckman-Spinco, Palo Alto, Calif., U.S.A.) equipped with a Bio-Cal Model AS-20 multi-sample programmer (Bio-Cal, Richmond, Calif., U.S.A.)[10]. The standard long column was packed to a column height of 54 cm with Beckman resin type AA-15. A stepwise four-buffer elution program, which is related to the system described by Guire *et al.*[11], was devised to separate all the components. The preparation and composition of buffers are given in Table I and the elution program in Table II. Flow-rates were maintained at 70 ml/h for the buffers and 35 ml/h for the ninhydrin. The ninhydrin reagent was prepared as described in the Beckman manual except titanous chloride was substituted for stannous chloride.

TABLE I

BUFFER COMPOSITION

Sodium citrate Beckman concentrates were diluted 1:10. Pentachlorophenol (0.4 ml of stock solution)[10] was added per 4 l of solution as an anti-mold agent. Sodium ion concentrations were adjusted by the addition of crystalline NaCl. Methylcellusolve and *n*-propanol were added as indicated and the final pH values were adjusted with either concentrated HCl or 50% NaOH as required.

Buffer	*Beckman concentrates*		*Final composition*		
	[Na^+]	*pH (25°)*	*NaCl (g/4 l)*	*pH (25°)*	*Organic solvent (vol %)*
A	2.0 *N*	3.25 ± 0.01		2.83	2% Methylcellusolve
B	2.0 *N*	3.25 ± 0.01	7.0	3.32	
C	2.0 *N*	3.25 ± 0.01	70.5	3.89	
D	3.5 *N*	5.26 ± 0.02	183.2	5.09	16% *n*-Propanol

Determination of color factors

The concentration of stock solutions of purified glc-gal-hyl and gal-hyl was determined from quantitative analysis of both hexose and hydroxylysine. Analysis of glucose and galactose was performed on the Technicon automated sugar analyzer (Technicon, Tarrytown, N.Y., U.S.A.) according to the method of Lee *et al.*[12] after hydrolysis with 2 *N* H_2SO_4 for 4 h at 100°. Analysis of hydroxylysine was performed on the amino acid analyzer using the procedure described by Guire *et al.*[11] after hydrolysis with 6 *N* HCl for 24 h at 105° in sealed tubes. A standard mixture was prepared from the glc-gal-hyl and gal-hyl solutions, a standard mixture of 18 amino acids (Pierce, Rockford, Ill., U.S.A.) and standard solutions of hydroxyproline, hydroxylysine, and methionine sulfoxide. The color yields of glc-gal-hyl and gal-hyl relative to leucine are 0.87 and 1.05, respectively.

TABLE II

ELUTION PROGRAM

Buffer	*Pumping time (min)*	*Run time (min)*	*Column temp.* (°C)*
A	40	0– 40	55
B	60	40–100	67
C	60	100–160	67
D	60	160–220	67
NaOH	11 (regeneration)	220–236	67
A	60 (equilibration)	236–286	55

* The column temperature reaches 67° 35 min after buffer B has been started, and returns to the initial 55° after the regeneration step has been concluded.

RESULTS AND DISCUSSION

Chromatographic system

Separation of methionine sulfoxide, glc-gal-hyl, gal-hyl and hydroxylysine from the amino acids usually present in alkaline hydrolysates of collagen or basement membrane was achieved on a single column of the Beckman amino acid analyzer as

shown in Fig. 2. In addition, leucine is well resolved, which permits its use as an internal standard since it is destroyed to the same extent as the hydroxylysine glycosides during alkaline hydrolysis[7]. The resolution of components was achieved with use of a stepwise four-buffer elution program (Tables I and II), which was controlled by a Bio-Cal AS-20 multi-sample programmer. The elution program is a modification of the program established for the automatic analysis of amino acids in hydrolysates of collagen and basement membrane[11].

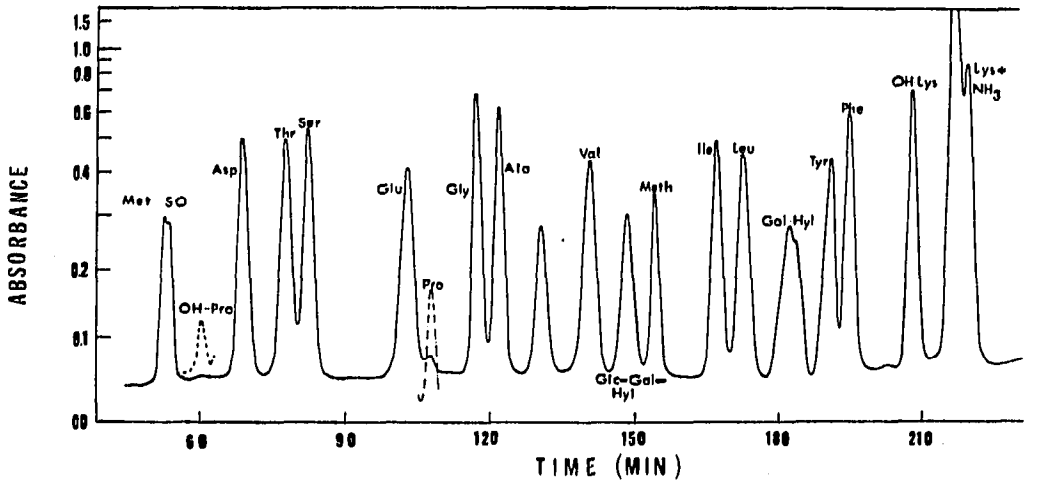

Fig. 2. Chromatogram of a standard mixture of amino acids, glc-gal-hyl, and gal-hyl on the Beckmall amino acid analyzer using type AA-15 resin. The concentration of components are 0.1 μmole for eacn amino acid, 0.07 μmole for glc-gal-hyl, and 0.08 μmole for gal-hyl. The stepwise four-buffer elutioh program is described under Experimental. The same chromatographic conditions are employed for an subsequent figures.

In the latter program glc-gal-hyl is also positioned between valine and methionine but gal-hyl is superimposed on the tyrosine and phenylalanine position. Gal-hyl was positioned between leucine and tyrosine by increasing the salt concentration, pH and pumping time of the C buffer. These alterations in program necessitated a lowering of salt concentration and increasing the pH of the D buffer. Gal-hyl could also be positioned between phenylalanine and hydroxylysine by the addition of propanol to the C buffer; however, an interfering peak appeared at this position in a thyroglobulin control sample. The absence of interfering peaks in the positions of glc-gal-hyl, gal-hyl and hydroxylysine for the elution program described in this paper is shown in Fig. 3. Thyroglobulin was chosen as a control since it does not contain the hydroxylysine glycosides but contains typical carbohydrates, which may give rise to interfering fragments. Increasing the salt concentration by a factor of 15 did not affect the separation of components given in Fig. 2. Hence, identical results were obtained for samples hydrolyzed in 2 *N* NaOH at a protein concentration of 1 mg/ml and 15 mg/ml.

A method for the measurement of hydroxylysine and its glycosides which also employs a single-column amino acid analyzer system appeared while this report was in preparation[13]. However, this procedure does not permit the simultaneous analysis

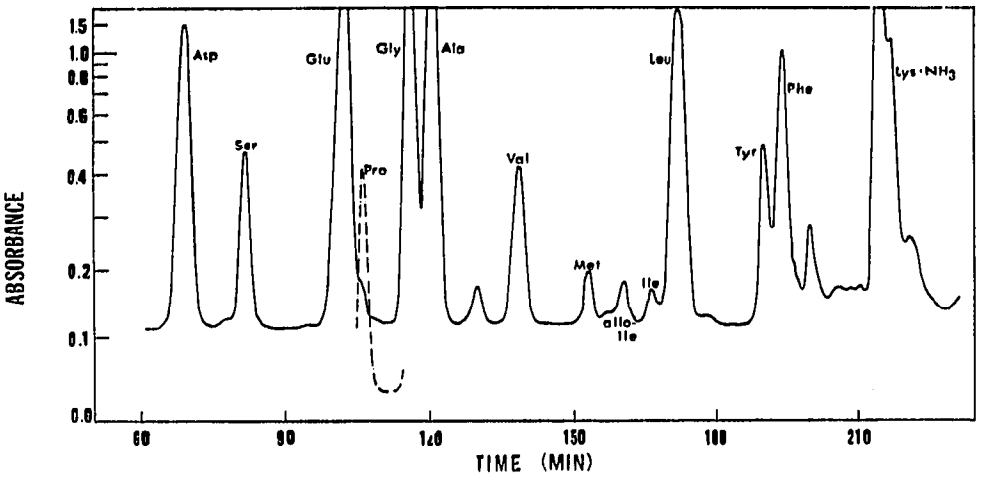

Fig. 3. Chromatogram of thyroglobulin (1.39 mg) alkaline hydrolysate.

of methionine sulfoxide along with hydroxylysine and its glycosides nor the use of leucine as an internal standard for absolute quantitation. In addition the glc-gal-hyl is not as well resolved from the other components as compared to the system described here and the effect of salt concentration in hydrolysates on the separation was not presented.

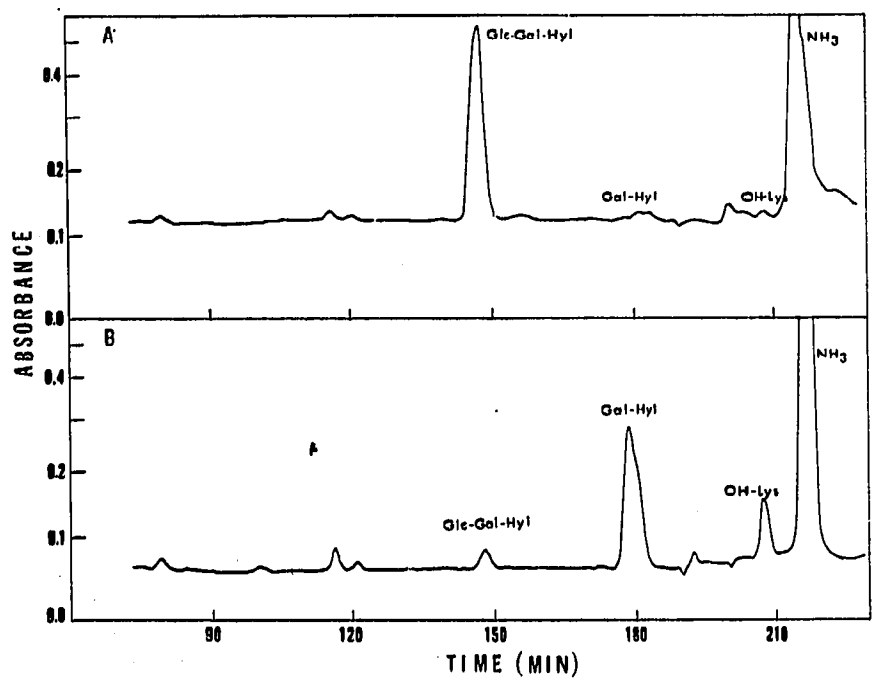

Fig. 4. (A) Chromatogram of glc-gal-hyl (0.15 μmole) from Bio-Gel P-2 column. (B) Chromatogram of hydrolysate from mild acid hydrolysis of 0.075 μmole purified glc-gal-hyl.

Purification of glc-gal-hyl and gal-hyl

A further purification of glc-gal-hyl and gal-hyl fractions from the Bio-Gel P-2 column (Fig. 1) was necessary in order to determine their color factors. Purification was achieved on a preparative scale using the analyzer system described above. A chromatogram of the Bio-Gel P-2 fraction of glc-gal-hyl is shown in Fig. 4A. The glc-gal-hyl is contaminated with small quantities of gal-hyl, free hydroxylysine, and other amino acids. A preparative run was made on the same column under the same chromatographic conditions and the peak containing glc-gal-hyl was collected and desalted on the Bio-Gel P-2 column (Fig. 1). The purified glc-gal-hyl was used to prepare a standard solution. Gal-hyl was prepared from the purified glc-gal-hyl by mild acid hydrolysis and purified on the analyzer in a similar fashion as described for glc-gal-hyl. A chromatogram of the gal-hyl acid hydrolysate is shown in Fig. 4B. The hydrolysate contained mainly gal-hyl but also unreacted glc-gal-hyl and free hydroxylysine.

Analysis of basement membrane and collagen

The chromatograms for basement membrane and collagen are shown in Figs. 5 and 6, respectively. Basement membrane was found to contain methionine sulfoxide, glc-gal-hyl, gal-hyl, and free hydroxylysine in the amounts of 3.9, 15.6, 0.2, and 5.7

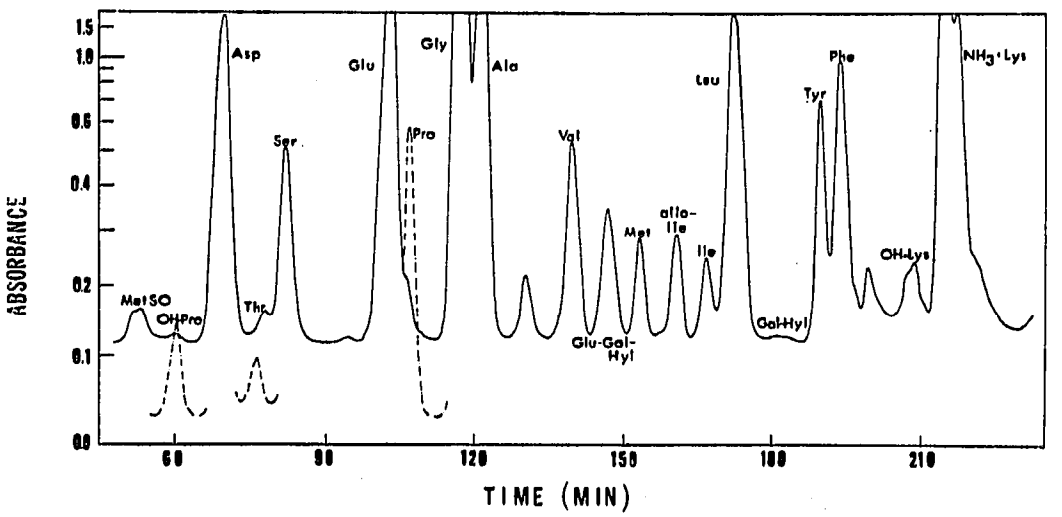

Fig. 5. Chromatogram of bovine glomerular basement membrane (2 mg) alkaline hydrolysate.

residues per 1000 amino acid residues based on the leucine content of the sample. These values are in close agreement with the literature values of 3.8, 16.8, 0.5, 5.2, respectively[1,5]. Collagen was found to contain methionine sulfoxide, glc-gal-hyl, gal-hyl and free hydroxylysine in the amounts of 0.21, 0.54, 0.50 and 5.4 residues per 1000 amino acid residues. These values for methionine sulfoxide and hydroxylysine closely agree with the literature values of 0.21 and 5.5, respectively[1,5], but the glc-gal-hyl and gal-hyl values are lower by a factor of two from the reported values of 1.24 and 1.06, respectively.

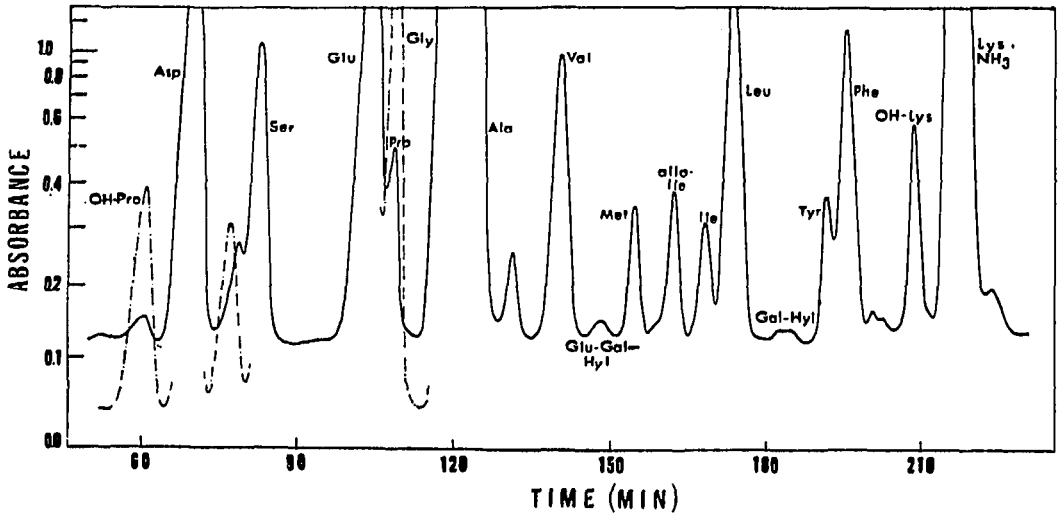

Fig. 6. Chromatogram of calf-skin collagen (2.7 mg) alkaline hydrolysate.

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