Journal of Chromatography, 145 (1978) 473—477
Biomedical Applications

CHROMBIO. 143

Note

Chromatography of glycosylated hydroxylysines and hydroxylysine on Dowex 50 resins of different degrees of cross-linking

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(Received December 29th, 1977)

Determination of the excretion rates of hydroxylysine (Hyl) and its glycosylated derivatives glucosylgalactosylhydroxylysine (GlcGalHyl) and galactosylhydroxylysine (GalHyl) has been shown to be useful in assessing the activity of diseases which affect collagen metabolism [1-3]. We have developed a method for measurement of the urinary excretion of GlcGalHyl, GalHyl and Hyl which is based upon separation of these compounds by chromatography on Dowex 50W-X8, followed by determination of the Hyl present in that portion of the column eluate which contains each compound [4]. The separation of the individual glycosylated hydroxylysines and Hyl is excellent; however, all urine samples obtained from normal individuals and from patients with a variety of diseases contain chromogenic material which is eluted from the chromatographic column prior to the elution of GlcGalHyl. This material exhibits chromatographic heterogeneity and is designated Fraction I (Fr I). The separation of this material from GlcGalHyl on Dowex 50W-X8 is adequate but the estimation of GlcGalHyl present in pooled eluates from calibrated chromatographic columns [4] would be more reliable if the separation of Fr I from GlcGalHyl could be improved without sacrifice of the separation of the other compounds of interest. This paper describes a modified chromatographic method which achieves this goal.

EXPERIMENTAL

Standard solutions

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Hydroxylysine (mixed DL and DL-allo) was purchased from Sigma (St. Louis, Mo., U.S.A.). GlcGalHyl and GalHyl were prepared from bovine lens capsule (Pel-Freez Biologicals, Rogers, Ariz., U.S.A.) collagen by the method of Spiro [5]. A 24-h urine sample obtained from a patient with Paget's disease was used as a reference urine sample to compare the different Dowex 50W resins. Aliquots of this urine sample were stored at —20° until chromatographed.

Reagents for column chromatography

Dowex 50W resins having different degrees of cross-linking were purchased from either Sigma (8% cross-linked) or Bio-Rad Labs., Richmond, Calif., U.S.A. (2% and 4% cross-linked). All resins were 200—400 mesh and were washed according to the procedure of Moore and Stein [6]. The washed resins were equilibrated with 0.1 *M* citrate buffer, pH 3.42 [6]. The eluting buffer contained 69 g citric acid monohydrate, 96 g sodium citrate dihydrate and 8 g disodium ethylenediamine tetraacetate in a final volume of 8 l. The pH of buffer was adjusted to 5.00 with 10 *M* NaOH (approximately 4.4 ml/l of buffer). This buffer is 0.08 *M* in citrate and contains 172 mequiv. Na/l. Following completion of each chromatogram the resin beds were washed with 0.2 *M* NaOH and then re-equilibrated with the 0.1 *M* citrate buffer, pH 3.42.

Chromatographic procedure

The following 24 × 1 cm ion-exchange columns were prepared: Dowex 50W-X8, Dowex 50W-X4, Dowex 50W-X2 and Dowex 50W-X8: Dowex 50W-X4 combined 1:1 (wet vol./wet vol.). The columns containing the combination of Dowex 50W-X8 and Dowex 50W-X4, designated combined-resin columns, were prepared either by layering the 4% cross-linked resin over the 8% cross-linked resin or by blending equal volumes (wet) of the two resins and then pouring the slurry into the chromatography column. Either 1 ml of a mixture containing GlcGalHyl, GalHyl and Hyl or a 1-ml aliquot of the reference urine, pH 2—3, was washed into the resin bed with the 0.08 *M* citrate buffer, pH 5.0. The chromatogram was developed at a flow-rate of 8 ml/h which was controlled by a syringe pump (Model 975 Infusion Pump, Harvard Apparatus Co., Dover, Mass., U.S.A.) fitted with adapters to hold four 60-ml plastic disposable syringes. The column effluents were collected in 1- or 2-ml fractions. Chromatography was carried out at room temperature. The eluate fractions were analyzed for the presence of Hyl and glycosylated hydroxylysines as described below.

Spectrophotometric analysis

Prior to spectrophotometric analysis, the glycosylated hydroxylysines and substances present in Fr I were hydrolyzed in order to release free hydroxylysine and Fr I chromogen(s) respectively. One ml of the column eluate fractions was placed in 100 × 13 mm culture tubes fitted with PTFE-lined screw-caps, and 1.0 ml of 4 *M* HCl was added to give a final concentration of 2 *M* HCl. The tubes were closed and hydrolysis was performed for 4 h at 105°. Following hydrolysis the solution was neutralized by adding 0.22—0.23 ml of 10 *M* NaOH to each ml of the hydrolysate solution. The volume then was adjusted to 5.0 ml

with 1.2 *M* Na_2HPO_4, 0.3 *M* citric acid buffer, pH 7.0 [7] and, if necessary, the final pH was adjusted to between 6.8 and 7.0 by the addition of either 2 *M* NaOH or 1 *M* HCl. Analysis for Hyl was then carried out as described by Blumenkrantz and Prockop [7]. Column eluate fractions which contained free Hyl did not require hydrolysis.

RESULTS

The elution positions of GlcGalHyl, GalHyl and Hyl standards were determined on each column. Table I summarizes the volumes of column eluate which separate Fr I, the glycosylated hydroxylysines and Hyl when 1-ml aliquots of the reference urine sample were chromatographed on Dowex 50W resins of varying cross-linking. The separation of GlcGalHyl from Fr I was improved by use of either a 4% or 2% cross-linked resin. However, there was a decrease in the separation of GlcGalHyl from GalHyl and of GalHyl from Hyl on the 4% cross-linked resin and a marked diminution in these two separations on the 2% cross-linked resin. The elution volume of the chromogenic material present in Fr I was not changed and the use of resins with fewer cross-linkages did not result in further fractionation of the substances present in this material. Analysis of the results obtained with the 8%, 4% and 2% cross-linked resins suggested that improved separation of GlcGalHyl from Fr I might be achieved by use of a 6% cross-linked resin without a significant decrease in the separation of the other compounds.

To our knowledge Dowex 50W-X6 resin is not commercially available, therefore chromatographic columns were packed with the 1:1 combination (wet vol./wet vol.) of Dowex 50W-X8 and Dowex 50W-X4. Fig. 1 shows the elution pattern obtained when 1 ml of the reference urine was chromatographed on a layered combined-resin column. There was no significant difference between the separations obtained on the layered combined-resin

TABLE I

SEPARATION OF FRACTION I, GLYCOSYLATED HYDROXYLYSINES AND HYDROXYLYSINE ON DOWEX 50W, 200—400 MESH RESINS WITH VARIOUS PERCENTAGES OF CROSS-LINKING

Cross-Linking (%)	Volume of eluting buffer between fractions (ml)*			Elution Volume of Hyl**
	Fr I—GlcGalHyl	GlcGalHyl—GalHyl	GalHyl—Hyl	
8	8	22	46	180
4	38	14	22	178
2	48	4	6	138
Combined-resin	26	20	34	176

*The results are expressed as the number of millimeters between the completion of the elution of each compound and the beginning of the elution of the following compound and are the average of two determinations.

**Volume of buffer required to complete the elution of Hyl.

columns and the blended combined-resin columns. Combined-resin columns were used over a period of months and no significant change in elution pattern of the substances present in the reference urine sample was observed during this period. Use of the combined-resin chromatographic columns resulted in improved separation of Fr I from GlcGalHyl, while excellent separation of GlcGalHyl from GalHyl and of GalHyl from Hyl was retained. The volumes of combined-resin column eluate which separate Fr I, the glycosylated hydroxylysines and Hyl are shown in Table I.

Although there was some variation, the volumes of eluate containing Fr I, the glycosylated hydroxylysines and Hyl were not significantly different during chromatography on resins of different degrees of cross-linking as compared to their elution volumes on Dowex 50W-X8. Urine samples which contain the amounts of glycosylated hydroxylysines and Hyl excreted by normal adults [1, 4] can be analyzed on calibrated chromatographic columns by pooling the eluate volumes previously demonstrated to contain GlcGalHyl, GalHyl and Hyl and by analyzing 1-ml aliquots (GlcGalHyl and GalHyl) or 2-ml aliquots (Hyl) of the pools [4].

Spectrophotometric analysis of those portions of the column eluate which contained Fr I and the glycosylated hydroxylysines without prior acid hydrolysis resulted in the detection of only trace amounts of chromogenic material. Acid hydrolysis of the column eluate fractions which contained Hyl did not affect the results of the spectrophotometric analyses of these fractions.

DISCUSSION

Separation of glycosylated hydroxylysines and Hyl on Dowex 50W-X8 is satisfactory; however, when the column eluates which contained GlcGalHyl, GalHyl and Hyl were pooled separately [4] and then analyzed, unexpectedly high values for GlcGalHyl occasionally were obtained. In these instances, further testing revealed that a portion of the Fr I chromogenic material had been included in the GlcGalHyl pool. Moore and Stein [8] demonstrated the effect of resin cross-linking on the chromatographic behavior of amino acids and polypeptides and noted that improved separations sometimes could be achieved by blending resins of different degrees of cross-linking. In order to

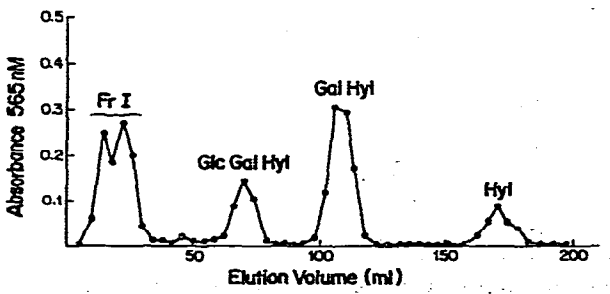

Fig. 1. The elution pattern of Fr I chromogens, GlcGalHyl, GalHyl and Hyl obtained when the reference urine was chromatographed on a layered, combined-Dowex 50W-resin column. Resin bed volume was 19.1 ml.

improve the separation of Fr I from GlcGalHyl without compromising the separation of the other Hyl-containing substances, we investigated the use of resins of different degrees of cross-linking. Among the types tested, this objective was best attained by use of a resin bed consisting of a 1:1 combination of 8% cross-linked Dowex 50W and 4% cross-linked Dowex 50W. Use of a combined-resin column has eliminated the problem of admixture of Fr I chromogen with the GlcGalHyl-containing portion of the column eluate.

The composition of the buffer was also a factor in the results obtained. Use of a 0.1 *M* citrate buffer, pH 5.0, which contained 182 mequiv. Na/l [4] to develop the combined-resin column resulted in good separation of Fr I from GlcGalHyl but the separation of GlcGalHyl from GalHyl was not as satisfactory as with the buffer described in this report. Less than optimal separation of all Hyl-containing compounds precludes the pooling of fractions collected from calibrated columns and measurement of the Hyl content of aliquots of the pools. If individual column eluate fractions are analyzed for Hyl, the original buffer does yield satisfactory separations and has the advantage of a shorter total running time since Hyl is eluted at 135—145 ml rather than at approximately 175 ml.

These investigations described in this report do not provide an indication of the nature of the substances present in Fr I. Six percent of the chromogen present in the Fr I component of the reference urine sample employed in this investigation is accounted for by the presence of peptide-bound hydroxylysine while the remainder of the chromogenic material is due to the presence of free and peptide-bound proline in this portion of the column eluate [4]. On the basis of the 16:1 ratio of proline to hydroxylysine in Fr I [4], we believe this material represents the collagen-related polypeptides which were described by Krane et al. [9]. Three percent of the Hyl present in the reference urine sample was present in Fr I. Askenasi has demonstrated that approximately 10% of the total urine hydroxylysine is present in these polypeptides [10, 11].

ACKNOWLEDGEMENTS

This investigation was supported by Clinical Investigation Grant CI-61 from the American Cancer Society and Public Health Research Grant HL-14212 (Pulmonary SCOR) from the National Heart and Lung Institute.

REFERENCES

1 J.P. Segrest and L.W. Cunningham, J. Clin. Invest., 49 (1970) 1497.
2 R. Askenasi and N. Demeester-Mirkine, J. Clin. Endocrinol. Metab., 40 (1975) 342.
3 R. Askenasi, M. DeBacker and A. Devos, Calcif. Tissue Res., 22 (1976) 35.
4 W.C. Bisbee and P.C. Kelleher, submitted for publication.
5 R.G. Spiro and S. Fukushi, J. Biol. Chem., 244 (1969) 2049.
6 S. Moore and W.H. Stein, J. Biol. Chem., 192 (1951) 663.
7 N. Blumenkrantz and D.J. Prockop, Anal. Biochem., 39 (1971) 59.
8 S. Moore and W.H. Stein, J. Biol. Chem., 211 (1954) 893.
9 S.M. Krane, A.J. Muñoz and E.D. Harris, Jr., J. Clin. Invest., 49 (1970) 716.
10 R. Askenasi, Clin. Chim. Acta, 59 (1975) 87.
11 R. Askenasi, V.H. Rao and A. Devos, Eur. J. Clin. Invest., 6 (1976) 361.