*Journal of Chromatography*, 76 (1973) 345–350


CHROM. 6370

# MICRO-CHROMATOGRAPHIC AND RADIOCHEMICAL ANALYSIS OF GLYCOPEPTIDES*

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(Received September 20th, 1972)

## SUMMARY

A sensitive radiochemical method for monitoring glycopeptides eluted from chromatographic columns was developed. The method, based upon the reduction of carbohydrates with sodium-[$^3$H]borohydride after hydrolysis of the glycopeptides, resulted in a hundred-fold increase in sensitivity over the anthrone method. The use of the reducing method to determine carbohydrates, in addition to the use of an analytical gel filtration column, permitted the resolution of milligram quantities of glycopeptides and yet 90% of the material remained for subsequent structural analyses.

## INTRODUCTION

Some of the more frequently used methods for detecting carbohydrates eluted during column chromatography (CC) are the anthrone, phenol–sulfuric acid, and orcinol reactions. All of these colorimetric methods are based on the formation of furfural derivatives by pentoses and hexoses in hot concentrated acid, which condense with an aromatic compound to produce a chromophore. These reactions are convenient to use because no prior hydrolysis of polysaccharides to monosaccharides is necessary and therefore the determinations may be made directly on column fractions as they are eluted. However, the limitations of these methods are that all require relatively large amounts of carbohydrates for each analysis (10–200 $\mu$g) and the various monosaccharides have different molar extinction coefficients.

As the investigations on the structures of the carbohydrate moieties of glycoproteins have progressed from those which are available in relative abundance to those present only in limited quantities, *i.e.* enzymes, hormones, etc., it has become

* Journal Article 2475 of the Agricultural Experiment Station, Oklahoma State University, Stillwater, Okla., U.S.A.
** Supported in part by Grants GB 24,291 from the National Science Foundation, AM 15112 and AM 10764 from The National Institutes of Health, and a Career Development Award (1 KO4 GM 42396) for K. E. EBNER.

necessary to develop methodology for monitoring CC separations of microquantities of glycopeptides with conservation of the separated glycopeptide material for use in subsequent structural analysis. This report is concerned with the development of such a method which is based on an existing radiochemical technique for the quantitative determination of microgram quantities of reducing monosaccharides[1].

## EXPERIMENTAL

### *Materials*

Sodium-[$^3$H]borohydride (800 mCi/mmole) was purchased from Amersham/Searle, Arlington Heights, Ill.; sodium borohydride from Fisher Scientific Co., Fair Lawn, N.J.; fetuin from Grand Island Biological Co., Grand Island, N.Y.; thyroglobulin was a gift from Dr. A. B. RAWITCH, Kent State University; and ovarian cyst fluid glycopeptide[2,3] was donated by Dr. DON M. CARLSON, Case Western Reserve University. Pronase, B grade, was obtained from Calbiochem, Los Angeles, Calif.; and Bio-Gel P-6 was purchased from Bio-Rad Labs., Richmond, Calif. N-acetylneuraminic acid was from Pfanstiehl Laboratories, Waukegan, Ill.; and ninhydrin (reagent grade) was from Pierce Chemical Co., Rockford, Ill.

### *Methods*

*Pronase digestion.* 5 mg each of fetuin, thyroglobulin, and ovarian cyst glycopeptide were separately dissolved in 0.2 ml of 0.2 *M* Tris–HCl buffer, pH 7.6, 0.0015 *M* in $CaCl_2$ (ref. 4). Approx. 250 $\mu$g of pronase were added and 1 drop of toluene was added to prevent bacterial growth. After 48 h at 37°, 250 $\mu$g of pronase were added and the digestion was continued for another 48 h. The tubes were placed in a boiling water bath for 10 min to stop the digestion, and aggregated protein was removed by centrifugation.

*Gel filtration chromatography.* A glass column, 0.4 (I.D.) × 100 cm, designed for analytical separations, was coated with Column Coat (Canalco, Rockville, Md.) before being filled to a height of 95 cm with Bio-Gel P-6, 100–200 mesh, which had been equilibrated in 0.1 *M* pyridine–acetate buffer, pH 5.0. The sample of pronase digest was applied in 0.2 ml and eluted at 22° with the equilibrating buffer. Fractions of 0.2 ml were collected at a flow-rate of 3 ml/h, and column flow was regulated with a LKB peristaltic pump, Model No. 4912A, in order to obtain fractions of constant volume. Tubes were capped with paraffin film and stored at −15° as soon as possible to minimize solvent evaporation.

*Analysis of column fractions by colorimetric methods.* Aliquots of the odd-numbered fractions were used for the determination of sialic acids by the thiobarbituric acid method of WARREN[5] and peptides by the ninhydrin reaction[6], and the entire even-numbered fractions were used for hexose determinations by the anthrone method[7]. In order to use these techniques for monitoring the chromatographic separation of these small amounts of glycopeptide, it was necessary to modify them for increased sensitivity. Therefore, for the WARREN reactions, 0.025 ml were transferred to 10 × 75-mm test tubes and the solvent was removed by lyophilization. Sialic acids were released in 0.05 ml of 0.1 *N* $H_2SO_4$ at 80° for 1 h. The volumes of all reagents were reduced to one-fourth and the standard curve was linear through 4 $\mu$g of N-acetylneuraminic acid. Amino acid and peptide determinations were made on 0.05 ml,

after 0.15 ml of water and 1.0 ml of ninhydrin reagent were added. The standard curve was linear through 0.04 $\mu$moles of leucine. In determining hexoses, 1 ml of anthrone reagent was added to each 0.2-ml fraction, and a linear standard curve was made through 40 $\mu$g of galactose.

All absorbances were measured with a Hitachi Perkin-Elmer Coleman 124 double-beam spectrophotometer in cells of 1-ml volume and 1-cm light path. These micro-modifications made the thiobarbituric acid method 4 times more sensitive, and the anthrone and ninhydrin methods were 5 times more sensitive.

*Analysis of column fractions by a radiochemical method.* For the determination of reducing equivalents, 0.025 ml of the even-numbered fractions were transferred with a microliter syringe to a screw-capped culture tube (Pyrex 9825) and the solvent was evaporated on a rotary evaporator (Evapomix, Buchler Instruments, Model 3-2100). Hydrolysis was done in 0.2 ml of 2 *N* HCl for 4 h at 100°. The HCl was removed on the rotary evaporator and 0.2 ml of 0.05 *M* sodium borate buffer, pH 10.4, were added to each tube.

0.05 *M* sodium-[$^3$H]borohydride in 0.1 *N* NaOH (6 $\mu$l), specific activity of $7.0 \cdot 10^6$ d.p.m./$\mu$mole, was added, the contents of the tubes were mixed well, the tubes were capped with teflon lined caps, and the reduction was run at 50° for 90 min. Then 50 $\mu$l of 2 *N* HCl were added to react with any remaining sodium borohydride, and the contents of the tubes were mixed well. The solvent was evaporated at 50° on the rotary evaporator. To the dry residue in each tube were added: 0.2 ml of 0.2 *M* pyridine–acetate buffer, pH 3.1, 0.2 ml of glucose-U.L.-[$^4$C] ($1 \cdot 10^4$ d.p.m./ml), 0.2 ml each of a 1:1 suspension (w:v) of Dowex 50 X4 [$H^+$], 200–400 mesh, and Dowex 1 X8 [formate], 200–400 mesh, in 0.2 *M* pyridine–acetate buffer, pH 3.1. The contents of the tubes were mixed well and allowed to stand for 10 min before the resin was centrifuged down. Samples of 0.1 ml were counted in BRAY's scintillation solution[8] for $^3$H and $^{14}$C.

Because sodium borohydride undergoes hydrolysis in aqueous solutions it was convenient to divide the prepared solution into 0.5-ml aliquots and to freeze them. Only as much reagent as was needed for the assay was thawed and the reagent was stored frozen for as long as two months without any losses.

## RESULTS

### *Radiochemical measurement of reducing equivalents*

The standard curve for the incorporation of tritium into ribose is shown in Fig. 1. Reduction was directly proportional to the amount of ribose in the range of 0.005–0.075 $\mu$moles and the slope of the line was $1.72 \cdot 10^6$ d.p.m./$\mu$mole of ribose. A greater excess of sodium-[$^3$H]borohydride can be added if greater amounts of carbohydrate are to be reduced, but in the present experiments the amount of borohydride relative to the sugar was kept low to minimize background counts due to non-volatile radioactivity after acidification of the reaction mixture. The reaction conditions described allow less than 0.005 $\mu$moles of reducing carbohydrate to be detected, which is a 20-fold greater sensitivity over the modified anthrone method and a 100-fold increase over the conventional anthrone procedure.

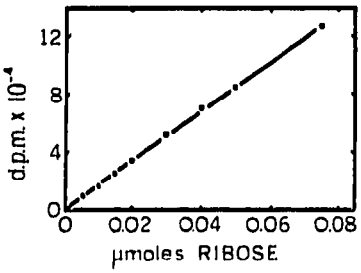


Fig. 1. Determination of carbohydrates as reducing equivalents with sodium-[$^3$H]borohydride. Ribose was used as a standard and the incorporation was $1.72 \cdot 10^6$ d.p.m./μmole of ribose. Experimental conditions are described in *Methods*.

*Detection of glycopeptides in the effluents of chromatographic columns*

Fetuin, thyroglobulin, and ovarian cyst glycopeptide were digested with pronase and chromatographed on Bio-Gel P-6 as described under *Methods*. Total carbohydrate was measured as reducing equivalents by the radiochemical method described. Also, the column effluent was monitored for sialic acid, hexoses, and peptides. The elution profiles are shown in Fig. 2. In each case the radioactivity from the measurement of reducing equivalents is coincident with the absorbance from sialic acid and hexose determinations. The recovery of reducing equivalents from the columns averaged about 90%.

The advantages of measuring glycopeptides by the radiochemical method is apparent since the method is 100-fold more sensitive than the anthrone method for detecting hexoses.

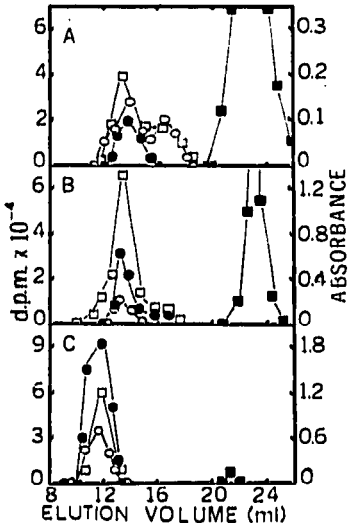


Fig. 2. Gel filtration on Bio-Gel P-6 of pronase digests of: (A), thyroglobulin; (B), fetuin; and (C), ovarian cyst glycopeptide. The elution diagrams show: □—□, carbohydrates determined as reducing equivalents; ○—○, hexose determined by the anthrone method; ●—●, sialic acids by the thiobarbituric acid method; and ■—■, amino acids and peptides by ninhydrin. Column size, 0.4 × 95 cm. Elution was performed with 0.1 *M* pyridine–acetate buffer, pH 5.0, at 22°. Fractions of 0.2 ml were collected.

The analytical column of Bio-Gel P-6 permitted better resolution of glycopeptides (Fig. 2) than columns of Sephadex G-25 and G-50 which have been used by other investigators[4,9]. The two carbohydrate units of thyroglobulin, one which contained sialic acid, were separated on the P-6 column but were not well separated on either Sephadex G-25 or G-50[4]. Likewise, two carbohydrate units were separated when digested fetuin was chromatographed, and both contain sialic acid. Although the existence of two carbohydrate units of fetuin has been suggested[9] their chromatographic separation has not previously been reported. The pronase digested ovarian cyst glycopeptide was eluted as a single peak in the void volume of the Bio-Gel P-6 column.

## DISCUSSION

The radiochemical measurement of carbohydrates as reducing equivalents with sodium-[$^3$H]borohydride developed by CONRAD *et al.*[1] has been modified and adapted to the detection of small amounts of glycopeptides in the effluents of chromatographic columns. The procedure allows for the sensitive detection of glycopeptides from the chromatography of pronase digests of glycoproteins and is at least a 100-fold more sensitive than the conventional anthrone method.

There is also some incorporation of $^3$H into peptides and amino acids probably caused by the reduction of disulfide groups by sodium borohydride[10]. This disadvantage of non-specific incorporation was overcome by maintaining the pH at 3.1 and adding a cation-exchange resin to remove the amino acids and peptides. Also, the sodium-[$^3$H]borohydride contains 1–3% of the radioactivity as non-volatile material after acidification but this could be reduced by 80% by the addition of an anion-exchange resin.

The high sensitivity of the reducing equivalents method along with the superior resolving capabilities of the analytical column used permits the chromatographic separation of glycopeptides of milligram quantities of glycoprotein and yet leaves about 90% of the material for other structural studies. Although the method was used in the analysis of glycopeptides in this case, it can be used equally as well in the structural studies of other complex polysaccharides.

A colorimetric method for the determination of carbohydrates which utilizes *p*-hydroxybenzoic acid hydrazide in alkaline solution appeared while this report was in preparation[11]. The method is technically simple and the sensitivity approaches that obtained in the present method. However, as with other colorimetric methods, the color yields are different for all sugars and the method was not applied to glycopeptide hydrolysates.

## ACKNOWLEDGEMENTS

The authors wish to thank Prof. H. E. CONRAD, University of Illinois, for technical advice, and Miss KATHRINE KRAFT for technical assistance.

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