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SENSITIVE METHOD FOR THE ANALYSIS OF CARBOHYDRATES BY GAS CHROMATOGRAPHY OF ^{3}H-LABELED ALDITOL ACETATES

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SUMMARY

A highly sensitive method has been developed for the analysis of carbohydrates from glycoproteins or lipopolysaccharides. The method is based on labeling the carbohydrates with [^{3}H]sodium borohydride, acetylating the resulting alditols and separating them by gas chromatography. The gas effluent is fractionated by trapping on silicone-coated glass beads and the amount of radioactivity is determined. This permits the quantitation of as little as 0.2 nmoles monosaccharide with an accuracy of 10 to 15%.

INTRODUCTION

The carbohydrate analysis of complex polysaccharides from biological sources requires a sensitive and accurate method of quantitation. The procedure which is used in many laboratories is based on the gas chromatographic (GC) separation of alditol acetates, and their detection by a flame ionization detector (FID)[1–3]. Although the sensitivity of the FID is very high, 0.05–1.0 mg of polysaccharide are necessary for a routine analysis. This amount is not available from many biological sources. Therefore investigators have improved the sensitivity of carbohydrate analysis by using ^{3}H-labeled alditols which were obtained by reduction of the polysaccharide hydrolysate by NaB^3H_4 (ref. 4). Separation of the labeled alditols was achieved by paper chromatography[5–8] or by high-voltage electrophoresis[9].

GC as the method of choice for the separation of ^{3}H-labeled alditols has not been used, because low amounts of ^{3}H-labeled compounds were difficult to quantitate in the gaseous effluent. Therefore we developed a method which combines the superior GC separation of alditol acetates with the high sensitivity of radioactive detection. The highest sensitivity of GC for radioactive substances can be obtained by trapping the separated components from the gas effluent[10]. This method has been applied to the detection of ^{14}C-labeled lipids[11–13]. We describe here a procedure for the GC analysis of radioactive carbohydrates with a high degree of sensitivity and accuracy. The method has been applied to the analysis of glycoproteins and lipopolysaccharides and permits the quantitation of neutral and amino sugars with 10–15% accuracy.

MATERIALS

[^{3}H]Sodium borohydride (specific activity 186 Ci/mol) was obtained from New England Nuclear (NET-023; Boston, Mass., U.S.A.); Aliquots of a 0.2 *M* solution in 0.1 *N* NaOH were kept at −20° for up to 2 months; sodium borohydride from Sigma (St. Louis, Mo., U.S.A.); D-xylose, D-galactose, D-glucose, D-glucosamine, and D-galactosamine from Calbiochem (Los Angeles, Calif., U.S.A.); N-acetyl-glucosamine and N-acetylgalactosamine from Pfanstiehl Labs. (Waukegan, Ill., U.S.A.) and fetuin from Grand Island Biological Co. (New York, N.Y., U.S.A.). *E. coli* K12 lipopolysaccharide was obtained by phenol–chloroform–water extraction of the bacteria[14]. GC was performed on a Packard gas chromatograph, Model 7400, equipped with an FID and a fraction collector, Model 851. Glass cartridges and filters were also obtained from Packard (Downers Grove, Ill., U.S.A.). 100-mesh glass beads were purchased from Potters (Carlstadt, N.J., U.S.A.); silicone oil DC710 from W. F. Nye (New Bedford, Mass., U.S.A.); the GC supports, 3% ECNSS-M on Gas-Chrom Q and 3% Poly A-103 on Gas-Chrom Q, from Applied Science Labs. (State College, Pa., U.S.A.). Liquid scintillation counting was done in Packard scintillation counters, Models 3320 or 3330, using 4 ml of a mixture of toluene (3800 ml) and Liquifluor (160 ml) (New England Nuclear).

METHODS

Hydrolysis

To 100 µl aqueous solution of 0.2–20 nmoles polysaccharide in a 6 × 50 mm test tube was added 5 µl of 12 *N* HCl to give a 0.5 *N* HCl solution[15]. The tube was sealed and heated overnight at 100°. After cooling the tube was opened and 1 µg of xylose (10 µl of 0.1 mg/ml) was added as internal standard.

Reduction

The solution was cooled in an ice bath and made alkaline by adding 100 µl 1 *N* NH_4OH. The alkalinity was tested by spotting 0.5 µl on pH-indicator paper. 2.5 µl of 0.2 *M* NaB^3H_4 in 0.1 *N* NaOH was added and the mixture was kept at 4° for 24 h[4]. The excess of borohydride was destroyed by addition of 10 µl acetic acid and the solution was brought to dryness in a desiccator. Boric acid was removed by addition of 50 µl methanol and evaporation. The addition and evaporation of methanol was repeated three times.

Acetylation

20 µl of pyridine and 20 µl of acetic anhydride were added to the dry residue. The tube was sealed and heated for 20 min at 100°. After cooling the sample was partitioned between 100 µl chloroform and 200 µl water. The chloroform layer was evaporated at 20° under a stream of nitrogen. The residue was dissolved in 10 µl chloroform and 5 µl were injected into the gas chromatograph.

Gas chromatography

Hexoses and heptoses were separated on a 3% ECNSS-M support on Gas Chrom Q[1]. Hexoses and hexosamines were separated on 3% Poly A-103 phase on

Gaschrom Q[16]. The retention times of the alditol acetates were determined by a pre-run of unlabeled standards and detection of the compound by the FID. For trapping the effluent components, the outlet of the column was connected directly to the fraction collector and $^1/_2$- or 1-min fractions were collected into tubes containing a filter plug and glass beads coated with 3% silicone oil DC710. Gas-tight connections were obligatory to give reproducible results. The glass beads were transferred from the tubes into counting vials and after addition of 4 ml to luene–Liquifluor mixture the radioactivity of the fractions was determined.

RESULTS

The application of GC to the analysis of ^{3}H-labeled alditol acetates should meet several requirements. The quenching of radioactivity by the silicone-coated glass beads must be negligible. The trapping of components from the carrier gas must be complete. Minute amounts of radioactive sugars must be separated as well as larger quantities of unlabeled alditol acetates. The reduction of the sugars by NaB^3H_4 must be complete. The background level of radioactivity must be low for the analysis of hydrolysates from glycoproteins or lipopolysaccharides. As described below, these requirements have been fulfilled by the method reported here.

Quenching of radioactivity by the silicone-coated glass beads

When a known amount of ^{3}H-labeled alditol acetate was counted in the absence and presence of the coated glass beads, there was no significant difference in the counts obtained. Thus the glass beads and the silicone oil, which do not interfere with the counting of [^{14}C]lipids[13], do not influence the counting efficiency of [^{3}H]alditol acetates and no further correction is necessary.

Efficiency of trapping

2000 cpm of ^{3}H-labeled xylitol acetate in 2 μl chloroform were injected into the gas chromatograph and the recovery of the radioactivity in the fractionated effluent was determined. The recovery was 1900–2000 cpm (95–100%) with a standard

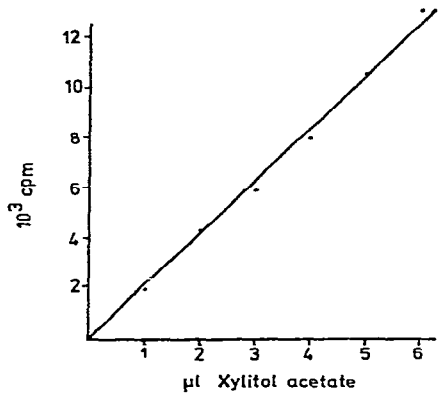

Fig. 1. Linearity of the recovery of radioactive xylitol acetate by GC. Increasing amounts of ^{3}H-labeled xylitol acetate in chloroform (1000 cpm/μl) were injected into the gas chromatograph and the recovery was determined by trapping the effluent.

deviation of 3.5%. Injection of increasing amounts of ^{3}H-labeled xylitol acetate gave a linear response of the recovered radioactivity (Fig. 1).

Separation of minute amounts of radioactive alditol acetates

When a mixture of ^{3}H-labeled alditol acetates was separated on a 3% ECNSS-M column, the retention times of the individual components coincided with those of unlabeled alditol acetates detected by FID (Fig. 2).

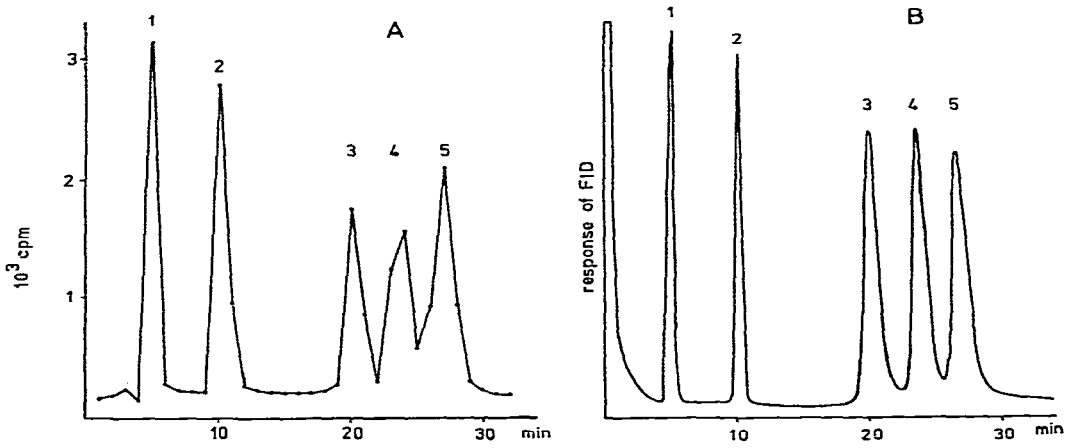

Fig. 2. Comparison of the detection and quantitation of carbohydrates by radio-GC trapping and the FID. (A) Separation of ^{3}H-labeled alditol acetates (0.22 nmoles each), as measured by trapping the effluent; fractions were collected in 1-min intervals. (B) Separation of unlabeled alditol acetates (20 nmoles each), as measured by the FID. Identical GC conditions were used for the separations. 1 = Fucose; 2 = xylose; 3 = mannose; 4 = galactose; 5 = glucose.

Reduction of sugars with NaB^3H_4

The conditions for complete reduction of carbohydrates with NaB^3H_4 have been determined previously[4].

Background level of radioactivity

To evaluate the background level of radioactivity 5 μg of fetuin and 10 μg of *E. coli* K12 lipopolysaccharide were analyzed. The radio gas chromatograms obtained (Figs. 3 and 4) showed that in this analytical procedure the background was sufficiently low that values for baseline correction can be readily established.

Calculation of results

The radioactivity of the individual peaks is calculated by adding up the counts of the respective fractions and subtracting the baseline radioactivity. As the radioactivity is incorporated into all alditols in equimolar amounts[4], the specific radioactivity of all ^{3}H-labeled alditols is identical and the molar amount of any component can be derived from its radioactivity relative to the internal standards.

Accuracy

The accuracy of the method was determined for the analysis of fetuin and

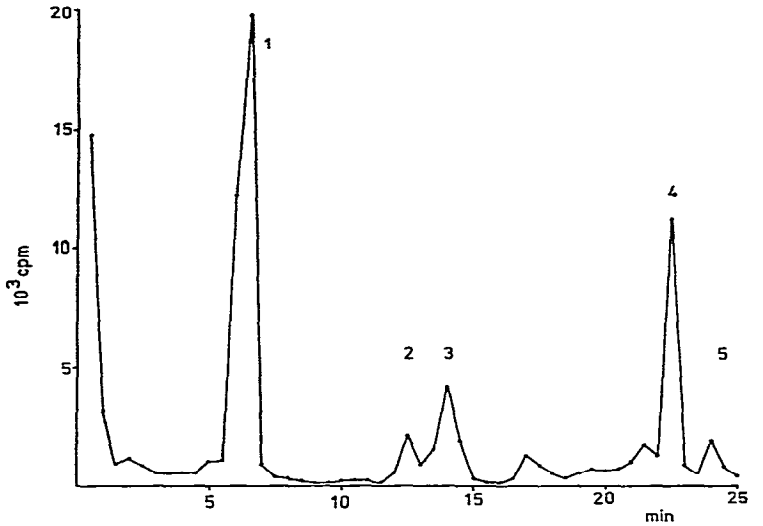

Fig. 3. Carbohydrate analysis of 5.0 μg of fetuin by GC separation of ^{3}H-labeled alditol acetates. Hexoses were separated on a Poly A-103 column at 200° for 16 min and hexosamines by raising the temperature to 240°. 1 = Xylose; 2 = mannose; 3 = galactose; 4 = glucosamine; 5 = galactosamine. 1 μg of xylose was added after hydrolysis of fetuin as internal standard. Fractions were collected at 1/2-min intervals.

E. coli K12 lipopolysaccharide (Table I). Four separate analyses of 5 μg fetuin and 10 μg *E. coli* K12 lipopolysaccharide gave a standard deviation of 10–15%. The analysis of 0.5 mg and 1.0 mg, respectively, by the conventional FID-detection method gave a standard deviation of 5–10%[1,3].

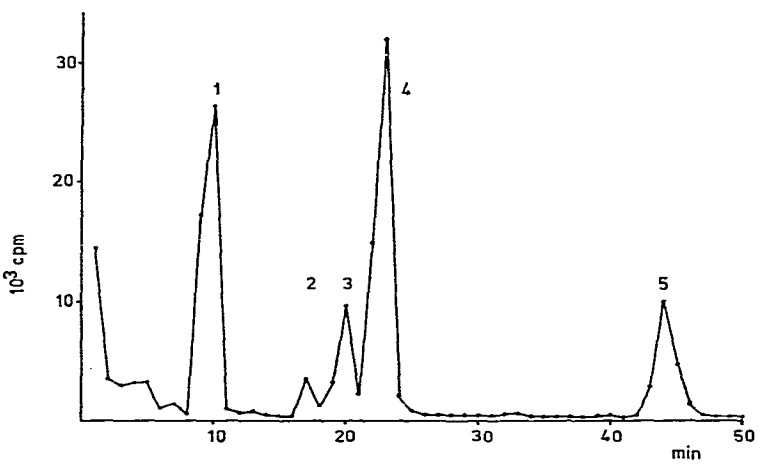

Fig. 4. Carbohydrate analysis of 10 μg of *E. coli* K12 lipopolysaccharide by GC separation of ^{3}H-labeled alditol acetates. Hexoses were separated on a ECNSS-M column at 180°, and heptose was eluted by raising the temperature to 195°. 1 = Xylose; 2 = mannose; 3 = galactose; 4 = glucose; 5 = heptose. 1 μg of xylose was added as internal standard after hydrolysis. Fractions were collected in 1-min intervals.

TABLE I

COMPARISON OF THE ANALYSIS OF CARBOHYDRATES OF FETUIN AND *E. coli* K12 LIPOPOLYSACCHARIDE BY THE CONVENTIONAL FID DETECTION METHOD AND THE RADIO-GC TRAPPING METHOD

0.5 mg of fetuin and 1 mg of LPS were analyzed by the conventional method[3]. 5.0 μg of fetuin and 10 μg of LPS were analyzed by the radioactive trapping method. Values are expressed as percent of weight.

Sugar component	*FID-detection (%)*	*Radio-GC (%)*
Fetuin		
Man	2.3	2.1
Gal	4.1	4.5
GlcN	6.0	6.1
GalN	1.0	1.1
LPS		
Man	0.85	0.85
Gal	2.8	3.2
Glc	11.1	11.1
Hep	4.4	4.8

DISCUSSION

Previous attempts to develop a highly sensitive method for the analysis of carbohydrates have led to the reduction of sugars to ^{3}H-labeled alditols and their separation by paper chromatography or paper electrophoresis[4–9]; however, a good separation of the alditols is very difficult by these methods[7,17]. Recently a method for highly sensitive carbohydrate analysis was described which is based on the GC separation of trifluoroacetyl derivatives and their detection by an electron capture detector[18], but its applicability to the analysis of polymeric carbohydrates has not been evaluated.

We have devised a GC method for the analysis of carbohydrates which allows the detection and quantitative determination of as little as 0.2 nmole of carbohydrates with an accuracy of 10–15%. The method is based on the labeling of carbohydrates with NaB^3H_4 which yields alditols having 25% of the specific radioactivity of the NaB^3H_4 used for reduction. The ^{3}H-alditols are converted into the volatile alditol acetates, separated by GC, trapped from the effluent, and the radioactivity of the fractions is determined. In the present analysis the lower limit of detection is determined by the specific radioactivity of the NaB^3H_4 which results in adequate amounts of radioactivity (*i.e.* 1500 cpm) in 0.1 nmole of alditol. This represents an increase of sensitivity of more than 100-fold over the quantitation by the FID[3]. This method of carbohydrate determination can be adopted to any mixture of alditol acetates which are separable by GC. We have also successfully applied the trapping of the gas effluent to the identification of partially methylated alditol acetates which were obtained by methylation analysis of polysaccharides.

This procedure is being employed for the analysis of the carbohydrates of viral glycoproteins.

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