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Microdetermination of molar ratios of neutral sugars on thin layers of borohydride-reduced cellulose*

An important problem in structural determination of the oligosaccharide units of glycoproteins is the availability of a sensitive, accurate and convenient analytical method for determining molar ratios of the constituent sugars. While paper chromatography of hydrolysates of oligosaccharides, followed by elution and colorimetric determination, can give good results[1,2], it is time consuming and requires relatively large amounts of material. Gas chromatography (GC) has also been used for quantitative determinations[3,4] and is extremely sensitive. However, unless the alditol acetate derivatives are prepared, one encounters problems with isomerization during sample preparation which could be serious if several different sugars are present. Thus, the preparation of suitable derivatives imposes a restriction on the sample size for GC analysis, even though a given analysis may require only 1% of the total sample. Recently, ion-exchange column chromatographic methods have been introduced[5,6] and have been found to give good separations with sensitivity in the 10 μg range. Both the GC and the ion-exchange methods require expensive instrumentation for quantitative results.

The method described in this communication requires only equipment for thin-layer chromatography (TLC) and for colorimetric determination. It combines the excellent separations of neutral sugars on thin layers of cellulose with a modification of the PARK–JOHNSON reducing sugar method[7] and enables the reproducible determination of molar ratios on as little as 0.5 μg of each sugar.

Materials and methods

Crystalline cellulose (Avicel SF, American Viscose Corp.) was reduced by a modification of the method of WOLFROM *et al.*[8]. The cellulose was suspended in 1.0 *M* aqueous sodium borohydride and vigorously stirred for 12 h. After this time, the once-reduced cellulose was filtered off and the above treatment repeated. The final reduced product was washed thoroughly with 0.1 *N* acetic acid and then with water and was dried in a desiccator. This treatment was found to yield a cellulose having chromatographic properties not significantly differing from those of native cellulose and a reducing content diminished to a point suitable for determination by the PARK–JOHNSON method. The dried, reduced cellulose was ground to a powder, and a slurry was prepared by homogenizing 5 g in 20 ml of distilled water in an Omnimixer blender. The slurry was rolled onto glass plates by hand, using a plastic rod. Plates prepared in this manner could be stored indefinitely with no special precautions regarding moisture or temperature and could be used with no pretreatment.

Standard sugars and neutralized hydrolysates of glycoprotein oligosaccharides were applied to the plates in 1–2 mm spots with a Hamilton microsyringe. The plates were developed to a distance of 10 cm. After development, the inner lanes were protected with aluminium foil and outer lanes were sprayed with *o*-aminobiphenyl reagent[9], and the plate was heated at 100° for 1 min to develop the fluorescent color. The sugars were removed from the plate by scraping free the area corresponding to

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the standard sugar and collecting it by suction into a glass-wool-plugged Pasteur pipette connected to a water aspirator. For a given plate, the same size areas were removed for each sugar. The reducing sugars were then eluted from the cellulose with distilled water and determined by the PARK–JOHNSON procedure scaled down to a final volume of 1.5 ml. In this modification, color development was linear with reducing sugar content over a range of 0.5–5 μg for D-mannose, D-galactose and D-xylose. Blanks for the determinations consisted of non-sugar containing areas of the plates.

Results and discussion

Blank values for the reduced cellulose layers were found to be low when the reduction was carried out as described. In an experiment in which ten areas were scraped from a plate to which no sample had been applied, the "plate blank" was found to give an absorbance of 0.112 $\pm$ 0.007 relative to water, as compared to a PARK–JOHNSON reagent blank of 0.085 $\pm$ 0.005. In the PARK–JOHNSON assay employed here, the color yield was $A_{600\ nm} = 0.267$ per μg reducing sugar as D-mannose against the reagent blank. Thus, when even as little as 0.5 μg of sugar is measured, the difference between the plate blank and the reagent is only 20% of the value for the sugar.

The recovery of reducing sugars from the reduced cellulose layers was found to be quantitative (Table I). Replicate aliquots of standard D-mannose solution were applied to a plate, chromatographed, eluted and determined colorimetrically. Using the same microsyringe and the same solution, the same volume of standard D-mannose solution was delivered to test tubes for direct colorimetric analysis. The results of both sets of reducing power determinations show that recovery of the standard sugar was virtually 100%. Recoveries were also shown to be quantitative when as much as 10 μg of neutral sugar was applied. In addition, mixtures of standard sugars were spotted on plates, separated, and recovered as described. The molar ratios of the isolated sugars did not differ significantly from those of the starting mixture.

TABLE I

QUANTITATION PARAMETERS FOR MICRODETERMINATION OF SUGARS ON REDUCED CELLULOSE LAYERS

	Volume of standard D-mannose solution (1 μg/μl)	*$A_{600\ nm}$ after Park–Johnson reaction*	*% recovery (A/B × 100)*
(A) Plate	1 μl (7 spots)	0.263 $\pm$ 0.006[a]	99
(B) Test tubes	1 μl (7 tubes)	0.267 $\pm$ 0.007	100.0
	Molar ratios relative to D-mannose as 1.00		
	D-Mannose	*D-Fucose*	*D-Xylose*
Standard sugars mixed	1.00	0.506	0.585
Standard mixture after TLC separation			
A	1.00	0.500	0.579
B	1.00	0.498	0.606

[a] After subtraction of plate blank.

TABLE II

MOLAR RATIOS OF GLYCOPROTEIN NEUTRAL SUGARS

Glycoprotein	*Neutral sugar constituent*	*Molar ratios of neutral sugar constituents*	
		TLC	*Automated borate chromatography*
α-Amylase	Mannose	3.90[a,b,c]	3.70
	Galactose	1.00	1.00
Bromelain II[d]	Mannose	2.00[e,f]	2.00
	Fucose	1.00	1.07
	Xylose	1.00	0.88
Bromelain III[d]	Mannose	2.00	2.00
	Fucose	1.03	1.12
	Xylose	0.93	0.95

[a] The neutral sugars in this glycoprotein show non-integral molar ratios due to microheterogeneity of the carbohydrate group[10].

[b] Ratios expressed relative to galactose as 1.00.

[c] Solvent system for separation was ethyl acetate–(isopropanol–water, 2:1), 60:40. Plates were developed twice.

[d] Commercial bromelain was fractionated by ion-exchange chromatography into several similar proteases with identical carbohydrate content.

[e] Ratios expressed relative to mannose as 2.00.

[f] Solvent system for separation was *n*-butanol–pyridine–ethyl acetate–acetic acid–water (10:3:3:3:4). Plates were developed three times.

The molar ratios of the major neutral sugar constituents of two glycoproteins, *Aspergillus oryzae* α-amylase and pineapple stem bromelain, determined by this method, agreed well with the ratios determined by automated borate complex anion-exchange chromatography[6]. The results (Table II) show that the present method gives values in good agreement with the sophisticated automated system.

In summary, microdetermination of molar ratios of reducing sugars by the present method provides an inexpensive valuable alternative to existing methods of quantitative sugar estimation.

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