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Note

Rapid quantitation of [^{3}H]fucitol and [^{3}H]mannitol by high-performance liquid chromatography: a convenient adjunct to borate electrophoresis for the microanalysis of carbohydrate in glycoproteins

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Takasaki and Kobata[1] developed a sensitive method for the determination of sugars commonly found in milk oligosaccharides which is based on quantitative labelling of sugars by reduction with NaB^3H_4 and separation of the sugar alcohols by borate electrophoresis. The original method was later modified for the analysis of glycoproteins by including an N-acetylation step and a deoxyribose internal standard[2]. Increased precision and reliability was obtained by the addition of ^{14}C-labelled sugars as internal standards, and by the inclusion of an ion-exchange step to remove peptides before reduction with NaB^3H_4 (ref. 3). All the neutral sugars commonly found in glycoproteins can be adequately separated in the form of alditols by borate electrophoresis except mannitol and fucitol[2,3]. The resolution problem is exacerbated by the fact that in most complex fucose-containing glycoproteins the mannose–fucose ratio is $\geqslant 3$. Because of this overlap of [^{3}H]mannitol and [^{3}H]fucitol it is not possible to determine their individual contribution to the total counts and hence the correct ratio of mannose to fucose without a further isolation step. This note describes their facile derivatization and separation by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Burdick & Jackson HPLC grade dioxane and hexane were purchased from Rainin (Brighton, Mass., U.S.A.). Benzoic anhydride and 4-dimethylaminopyridine were obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and Sep-pak reversed-phase columns from Waters Assoc. (Milford, Mass., U.S.A.). D-[1-^{14}C]Mannose (spec. act. 48.6 mCi/mmol), L-[1-^{14}C]fucose (spec. act. 59.2 mCi/mmol) and NaB^3H_4 (spec. act. 338 mCi/mmol) were purchased from New England Nuclear (Boston, Mass., U.S.A.). Standard sugars were supplied by Pfanstiehl (Waukegan, Ill., U.S.A.), and their crystalline alditols were prepared by reduction with $NaBH_4$.

Sample saccharide mixtures were reduced and analyzed by electrophoresis in 0.06 *M* borate as described[1]. A mixed sugar alcohol standard (5 μg each) was run in an adjacent lane and visualized with periodate–benzidine reagent[7]. Radioactive alditols were located by radioscanning and appropriate sections of the chromatogram

were cut into 0.5-cm strips, eluted with 0.5 ml water for 2 h and counted after the addition of 5 ml scintiverse (Fisher Scientific, Medford, Mass., U.S.A.).

Samples containing [^{14}C]mannose and [^{14}C]fucose in a ratio of 2.5:1 or 1:1 were reduced with $NaBH_4$ and benzoylated at 37° for 2 h in 0.5 ml of pyridine containing 10% benzoic anhydride and 5% 4-dimethylaminopyridine (DMAP). Following benzoylation, excess reagents were removed by one of two alternative methods: ("E") pyridine was removed under nitrogen and the sample was taken up in chloroform, extracted with 5% sodium carbonate and then with 0.05 *M* hydrochloric acid containing 5% sodium chloride[6]; or ("S") the sample was diluted with 4.5 ml of water and applied to a Sep-pak which was washed with 15 ml of water to remove benzoic acid and DMAP; benzoylated alditols were then eluted with 5 ml of methanol, taken to dryness and dissolved in carbon tetrachloride for HPLC. HPLC was carried out on a Zipax column (Dupont Instruments, Wilmington, Del., U.S.A.) using a Waters Assoc. liquid chromatography system as described[5]. Elution conditions are given in the legend to Fig. 2.

RESULTS AND DISCUSSION

While evaluating the procedure of Takasaki and Kobata[1] for the microanalysis of sugars in glycoproteins, difficulties were encountered in obtaining adequate resolution between [^{3}H]mannitol and [^{3}H]fucitol. This problem has since been reported by others[2,3]. A typical chromatogram is shown in Fig. 1, which shows the electrophoretic separation of a reduced and labelled mixture of standard sugars present in the same relative molar proportions as reported for human asialolactoferrin ($Fuc_1Gal_2Man_3GlcNAc_4$) (ref. 4). The measured ratios of galactose and N-acetylglucosamine are in close agreement with the theoretical values (Table I) and so is the sum of mannose and fucose; however, without a further isolation step, the relative amounts of these two sugars cannot be determined.

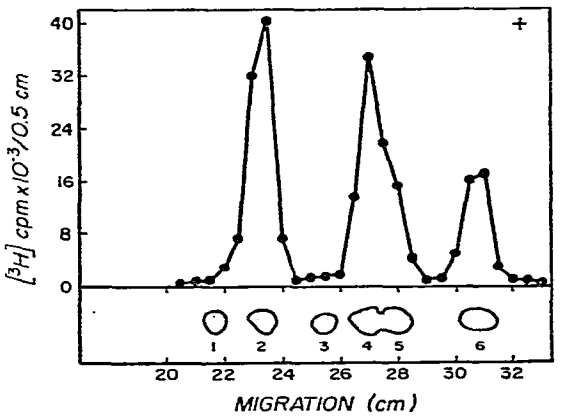

Fig. 1. Paper electrophoresis of alditols. Upper trace: a standard mixture containing Fuc (1 nmol), Gal (2 nmol), Man (3 nmol) and GlcNAc (4 nmol) was reduced with NaB^3H_4 and electrophoresed on Whatmann No. 3 MM paper in 0.06 *M* borate buffer at 60 V/cm for 1.5 h. Lower trace: unlabelled standard alditols (5 μg each) visualized with periodate–benzidine reagent[7]. 1 = N-Acetylgalactosaminitol; 2 = N-acetylglucosaminitol; 3 = glucitol; 4 = mannitol; 5 = fucitol; 6 = galactitol.

TABLE I

QUANTITATION OF [^{3}H]ALDITOLS BY BORATE ELECTROPHORESIS
See legend to Fig. 1 for experimental details.

Sugar	*Total cpm*	*cpm/nmol*	*Molar ratios (Gal = 2)*	
			Actual	*Measured*
GlcNAc	92,100	23,000	4	4.26
Man + Fuc	88,750	22,200	3 + 1	4.10
Gal	43,250	21,600	2	2.00

After perbenzoylation, fucitol and mannitol can be speedily separated by HPLC on an easily packed Zipax column by gradient elution with dioxane in hexane (Fig. 2). An isocratic separation could be employed with only a slight increase in analysis time. The elution of unlabelled carrier fucitol and mannitol is followed by monitoring the absorbance at 230 nm; appropriate fractions are collected and counted to obtain the ratio of fucose–mannose in the original sample. Alternatively, the *p*-nitrobenzoyl derivatives can be formed and monitored at 254 nm by an inexpensive fixed wavelength UV detector[6].

[^{14}C]Mannitol and [^{14}C]fucitol were used to check for any selective losses of either component in the HPLC analysis itself, or in the work-up prior to analysis by two different procedures designated as "S" and "E" in Table II. Both procedures gave comparable recoveries, mostly in the 90–95% range, and recovery from the column was essentially quantitative. The agreement between the experimentally determined and known ratios of [^{14}C]mannitol–[^{14}C]fucitol was excellent indicating that there was no selective loss of either component. The precision of the method is indicated by the excellent agreement between duplicate samples: (2.44 $\pm$ 0.04 S.D. and 0.97 $\pm$ 0.02). Further precision could doubtless be obtained by replicate injections of the same

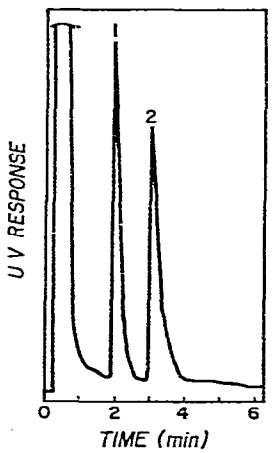

Fig. 2. HPLC separation of fucitol and mannitol (after perbenzoylation). Column: Zipax (2.1 mm × 50 cm); solvents: A, hexane; B, 20% dioxane in hexane. A gradient of 2% to 15% B in A was run in 5 min at 2 ml per min. UV absorption at 230 nm was recorded. Identification of peaks: 1 = fucitol; 2 = mannitol.

TABLE II

QUANTITATIVE EVALUATION OF THE RECOVERY OF ALDITOLS FOLLOWING DERIVATIZATION AND HPLC

Starting ratio of counts [^{14}C]mannitol–[^{14}C]fucitol	*Clean-up procedure**	*Recovery after benzoylation and clean-up (%)*	*Recovery from HPLC (%)*	*Measured ratio of counts [^{14}C]manniol–[^{14}C]fucitol***
2.5	S	93	94	2.40
	S	66***	110	2.41
	E	96	101	2.48
	E	61***	109	2.47
1.0	S	99	105	0.94
	S	93	107	0.98
	E	92	101	0.98
	E	108	78	0.97

* Following benzoylation, excess reagents were removed either by solvent extraction (E) or by a Sep-pak reversed-phase column (S) as described under Experimental.

** Fractions corresponding to the UV elution profile of carrier mannitol and fucitol were collected, evaporated to dryness and counted.

*** Recovery of these particular samples was lower than usual. Nevertheless, the measured ratio of [^{14}C]mannitol–[^{14}C]fucitol was not affected by this.

sample. For use of this procedure in the analysis of glycoproteins the sample must be subjected to borate electrophoresis prior to HPLC, since galactitol and glucitol cannot be separated from mannitol using the conditions reported here.

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