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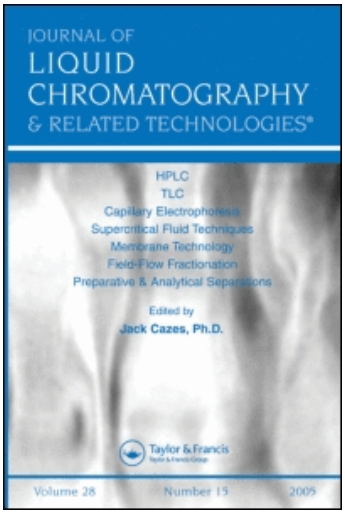

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THE RAPID DETERMINATION OF NEUTRAL SUGARS IN BIOLOGICAL SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A procedure for the analysis of neutral sugars in biological specimens is described. The method entails acid hydrolysis of the sample to liberate monosaccharides, which are subsequently derivatized with dansyl hydrazine. The sugar-dansyl hydrazones are separated and quantitated by hplc on a 5μ C18 RadialPak column with a gradient of acetonitrile in 10mM ammonium sulfate at pH 7. Fluorescent detection of the derivatized sugars permits 100-fold increased sensitivity compared to previously published glc methods.

This procedure was applied to the neutral sugar analysis of a glycoprotein of known composition (thyroglobulin) and to hard keratin fibers. The latter substance served as a model to critically evaluate the method on a highly resistant biological matrix containing low concentrations of neutral sugars.

INTRODUCTION

Analysis of carbohydrates in biological materials is a demanding and time-consuming task. Classically, gas-liquid chromatography (glc) has been used for carbohydrate analysis of glycoproteins. Since sugars are not volatile, they must first be converted to suitable volatile derivatives. These techniques uti-

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lize alditol acetates (1), aldonitrile acetates (2), or trimethylsilyl ethers of methyl glycosides (3), and are lengthy procedures to complete. Liquid chromatography on ion-exchange columns with post-column derivation (4-6) has also been used. While these procedures provide adequate sensitivity for the detection of 0.1 μg of carbohydrate, they are complicated by the necessity for large quantities of starting material, cumbersome volumes of hydrolytic and pretreatment solutions and, in many cases,multiple peaks from discrete sugar residues.

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A recent technique (7) employs 5-dimethylamino-naphthalene sulphonyl (dansyl) hydrazine to selectively label reducing sugars and the resulting derivatives are separated by high-performance liquid chromatography (hplc). This procedure is demonstrably more rapid and sensitive than earlier glc or hplc methods. In the present communication, we have applied this method to the analysis of neutral sugars of a characterized glycoprotein (thyroglobulin), and to human hard keratin fibers. We show that this is the method of choice for neutral sugar analyses of biological materials.

MATERIALS AND METHODS

Solvents, standards, and hplc equipment have been described previously (7). Acetonitrile was purchased fresh and stored at 4°C. Bovine thyroglobulin (Type I) was purchased from Sigma Chemical Co. (St. Louis, MO). Human hard keratin fibers were from a single donor. The anion-exchange resin was AG1x8 (acetate

form) 200/400 mesh from Bio-Rad Laboratories (Richmond, CA). Before use, the resin was converted to the carbonate form by washing first with 2N sodium carbonate (200 ml), then with water (200 ml). For cation-exchange chromatography, AG50Wx8 (hydrogen form) 200/400 mesh resin (Bio-Rad) was used after purification. The resin was purified by converting it first to the sodium form and then back to the hydrogen form (8). This treatment removed an unidentified yellow substance (probably a resin degradation product), which interfered with the hplc separation. The final product was suspended in two volumes of water and 5ml was pipetted into a 1 x 30 cm chromatographic tube.

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Glycoproteins and keratin fibers were hydrolyzed with 1N or 2N HCl at 100°C for various times (see Figures 2 and 4). After cooling, maltose was added as an internal standard. The sample was neutralized by adding an excess of AG1 resin (carbonate form, 0.5g per equivalent H+) and then transferred to a 1 x 30 cm chromatographic tube, whose outlet fed into the cation-exchange column. After rinsing the columns with 10ml water, the effluent was dried on a rotary evaporator, then redissolved in a small volume of water.

The previously described procedure (7) for the hplc analysis of reducing sugars was modified as follows. The sugar-dansyl hydrazone derivation product (40 μl) was diluted only 7.5-fold and used without Sep-Pak treatment. For increased resolution, separations were performed on a RadialPak 5μ C18 cartridge (0.8 x 10 cm) with a 2 ml/min flow rate and a concentration

gradient of acetonitrile (solvent B) in 10mM ammonium sulfate at pH 7. (The gradient was isocratic at 22%B for 8 min, increase linearly in 9 min to 28% B, step to 50% B for 5 min.) These modifications optimized the separation of the neutral sugars commonly found in mammalian glycoproteins.

RESULTS

A typical separation of standard sugar-dansyl hydrazones is shown in Figure 1. The procedure provided baseline resolution of nearly all derivatives over a twenty-fold range of sugar concentrations. Dansoic acid, a by-product of the derivation reaction, appears at the front of the chromatogram. An unidentified contaminant from the derivation reagent elutes after xylose. It is sufficiently resolved so as not to hamper the quantitation of xylose. Unreacted dansyl hydrazine is removed from the column with 50% acetonitrile, and elutes after fucose (not shown).

Separation of the sugar derivatives from a hydrolysate of thyroglobulin is shown in Figure 2. With our sample pretreatment scheme, the hexoses elute with no interference. If preparative ion-exchange chromatography is omitted, additional unidentified peaks appear which elute near glucose and complicate the results (data not shown).

Recovery of neutral sugars from thyroglobulin hydrolysates is shown in Figure 3. Four hours hydrolysis produced linear recoveries of each hexose independent of the glycoprotein concentration. Fucose, however, required a shorter hydrolysis

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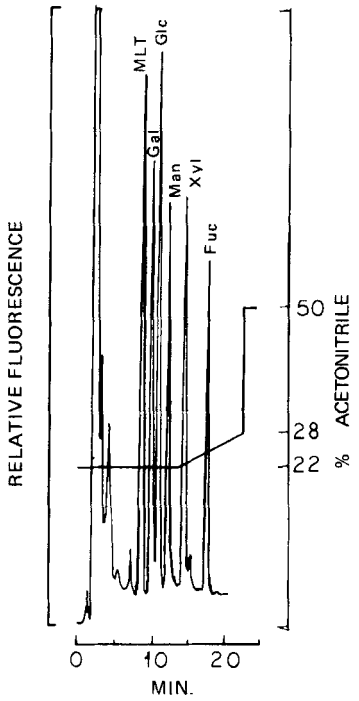

FIGURE 1. Chromatographic separation of standard sugar-dansyl hydrazones. Conditions for separation as described in text. Detector was Schoeffel FS970 Spectrofluoro Monitor (excitation wavelength, 240nm; emission longpass filter, 550nm; range, 0.2 μAFS; sensitivity, 42%; time constant, 2.5 sec). Sample was 1 nmole each standard: mlt, maltose; gal, galactose; glc, glucose; man,mannose; xyl, xylose; fuc, fucose.

time (1 hour) to obtain a linear recovery. The concentration of each sugar in this thyroglobulin sample is given by determining the slope of the appropriate curve (Table 1).

To demonstrate the sensitivity and versatility of our method, it was applied to the analysis of neutral sugars in

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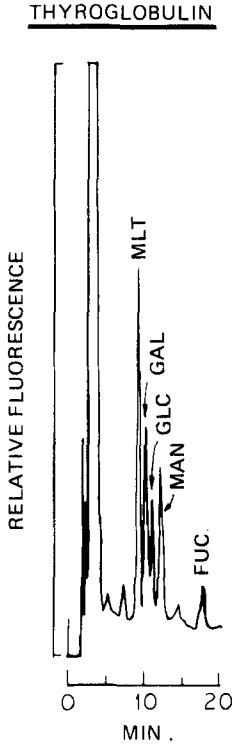

FIGURE 2. Sugar dansyl hydrazones from a thyroglobulin hydrolysate (4 hr at 100°C in 2 ml 1N HCl). Conditions as described in Figure 1.

hard keratin fibers. Human hair provided an ideal model for the analysis of small amounts of sugar in a resistant biological matrix. Figure 4 shows a chromatogram of the sugar-dansyl hydrazones produced from a keratin hydrolysate. Even though the sugars were present in very low concentrations in keratin fibers, there was still no observable interference from peptide or melanin by-products formed during hydrolysis.

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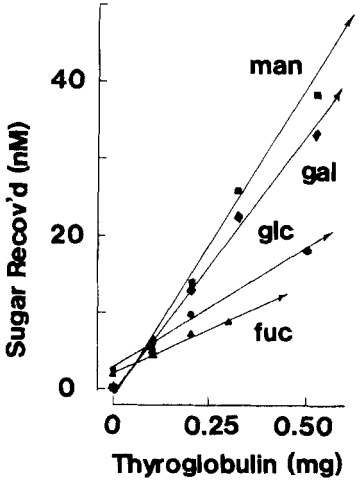

FIGURE 3. Proportional recovery of neutral sugars from various amounts of thyroglobulin hydrolyzed in 1 ml 1N HCl at 100°C (fucose for 1 hr., remainder for 4 hr.) Each point is the mean of 3 determinations.

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TABLE 1.

Concentration of Neutral Sugars in Bovine Thyroglobulin

Saccharide	Concentration	
	Found *	Reported **
	(μmoles per g)	
Galactose	66.4	72
Mannose	79.9	128
Fucose	23.1	24
Glucose	31.4	--

* - from the slopes of the curves in Figure 3 by linear regression analysis

** - Reference (9)

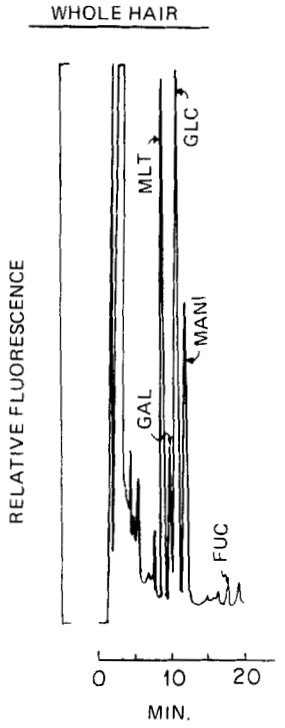

FIGURE 4. Separation of the sugar-dansyl hydrazones from a hard keratin fiber hydrolysate (2 hr at 100°C in 2 ml 1N HCl). Chromatographic conditions as described in Figure 1.

The kinetics of sugar release from keratin fibers is shown in Figure 5. Maximal release was obtained at 2 hours. Hydrolysis in 2N HCl was more effective in releasing saccharide from keratin than lower concentrations of HCl (data not shown). As with thyroglobulin, recovery of sugars from the hydrolysates was linear and independent of keratin concentration (Figure 6). The sugar content of hard keratin fibers was readily determined from the slopes of these curves (Table 2).

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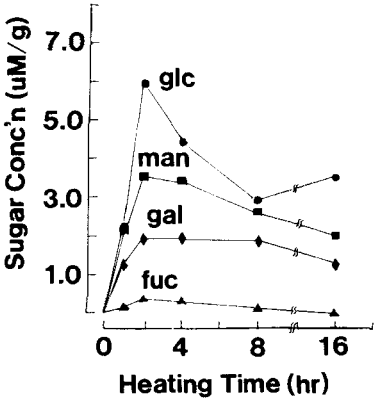

FIGURE 5. Rate of hydrolysis of neutral sugars from hard keratin fibers in 2 ml 2N HCl at 100°C. Analyzed as their dansyl hydrazones in triplicate; chromatographic conditions as in Figure 1.

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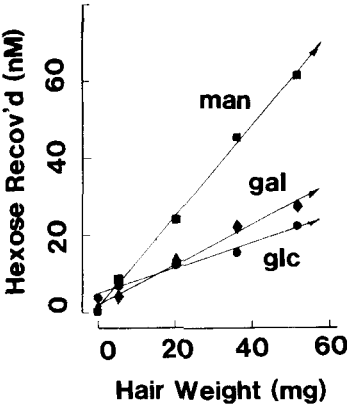

FIGURE 6. Proportional recovery of neutral sugars from human hard keratin fiber hydrolysates (2.5 h at 100°C in 2 ml 2N HCl). Each point is the mean of 3 determinations.

TABLE 2.

Concentration of Neutral Sugars in Hard Keratin Fibers

Saccharide	Concentration *
	(μmoles per g)
Galactose	0.53
Mannose	1.18
Glucose	0.34

* - from the slopes of the curves in Figure 6 by linear regression analysis

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DISCUSSION

In a previous manuscript, we demonstrated that neutral sugars commonly found in mammalian tissues can be separated and quantitated by hplc of their dansyl hydrazone derivatives (7). This method offers greatly increased speed, versatility, and sensitivity of detection over previously published methods (1-6). Glc methods for sugar analysis currently in use (1-3) are some 100-fold less sensitive than our present method and require many more hours to complete (7). The versatility of our method is demonstrated by noting that previous methods of analysis require mg amounts of glycoprotein starting material (1-4), whereas we characteristically use 10μg of glycoprotein for hydrolysis and derivation. Trace determinations (e.g. in keratin sugar analysis) can accomodate several mg of protein without interference.

In the present communication, we have improved the resolution of galactose, glucose and mannose dansyl hydrazones by using a 5μ C18 column. In conjunction with a specific gradient of acetonitrile, the 5μ C18 column also gave improved efficiency for xylose and fucose. These late eluting sugars can now be quantitated with even greater sensitivity and accuracy than was previously reported (7).

It is worthwhile noting that the integrity of the acetonitrile is crucial to the success of our method. By storing the solvent at 4°C, we have insured its integrity for periods in excess of 6 months. When stored at room temperature for periods more than a few weeks, deleterious effects on our chromatographic separations were repeatedly observed. While we have not investigated the source of this effect, it seems likely that a decomposition product from the acetonitrile alters the retention of a derivation by-product and thereby disrupts the chromatogram. We have observed that the effect is usually reversible, but on one occasion has irreversibly altered the stationary phase.

Thyroglobulin served in the critical evaluation of this procedure for glycoprotein analysis. The linear correlations obtained in Figure 3 demonstrate that this method provides quantitative results. Galactose and fucose were found (Table 1) in concentrations similar to reported values for calf thyroglobulin, while mannose gave two-thirds of the reported levels (9). If hydrolysis conditions were optimized, the mannose content may reach reported values. However, after one hour hydrolysis, we

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have recovered 2/3 of the mannose liberated in four hours of hydrolysis. This suggests that we are approaching the optimal hydrolysis time with one hour heating. More likely, this preparation of thyroglobulin probably contains a lower concentration of mannose. Since thyroglobulin does not normally contain glucose (9), the presence of a significant amount of glucose indicates the presence of contaminants in the commercially available thyroglobulin sample.

In the quantitative carbohydrate analysis of any sample, conditions must be optimized for the hydrolytic release of each component sugar. The different hydrolysis times required for the hexoses and fucose is probably related to the lability of fucose to acid hydrolysis. In fact, the bimolecular nature of likely fucose degradation pathways supports the concentration-dependency observed for its recovery after four hours of hydrolysis (10).

Hard keratin fibers present a challenging subject for carbohydrate analysis. The tenacity of the keratin protein structure, its low carbohydrate content, and the presence of various pigments introduce special complications. However, we have shown that the application of this procedure overcomes these obstacles without difficulty. Indeed, the results demonstrate that ours is the method of choice for the analysis of neutral sugars in biological materials.

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