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THE RELATIVE SENSITIVITY OF VARIOUS REAGENTS FOR THE DETECTION AND DIFFERENTIATION OF SUGARS AND SUGAR DERIVATIVES IN GLYCOPROTEINS

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SUMMARY

Eleven sugars reported to be found in the carbohydrate moiety of different glycoproteins were separated by paper chromatography in *n*-butanol–pyridine–water. A study was made of the relative sensitivity and specificity of twelve colouring reagents for the detection of each sugar and sugar derivative. A distinction between those reagents dependent on the reducing property of the sugar and those involving at one stage or another a condensation reaction is suggested.

INTRODUCTION

Many investigators reported on the composition of the carbohydrate moiety of glycoproteins[1,2] using a variety of techniques for hydrolysis. Others[3,4] demonstrated that not all sugars were released at the same rate and some degraded during hydrolysis. Thus the quantitative results for the carbohydrate composition of glycoproteins may be questioned. As a result, a series of experiments was designed to investigate the effect of hydrolysis on the individual sugars of the glycoproteins. The present paper reports on the sensitivity and specificity of various reagents for visualizing sugars and sugar derivatives separated by paper chromatography.

In choosing from the many colour reagents available, three interrelated factors were considered; sensitivity, specificity and the resolution of the various sugars by the chromatographic system used. The reagent had to be sufficiently sensitive to detect concentrations below that which would overload the chromatographic system. Also the relative sensitivity for each sugar and sugar derivative was of importance in establishing the limits of the reported qualitative and quantitative composition. The fact that certain reagents were specific for an individual sugar or a chemically related group of sugars does not necessarily mean that they were effective at all levels applied.

Of the reagents described in the literature, those with reported sensitivity in the 1–20 μg range were selected[5–13]. Data on the sensitivity of a specific method for a number of sugars and sugar derivatives were found in two of these papers[8,9] but none compared the sensitivity of several methods for a specific sugar. Both aspects were determined in one solvent system for sugars reported to be present in chondroitin sulphate and other polysaccharides associated with proteins.

EXPERIMENTAL

All chemicals were analytical reagents used without further purification. Two standard solutions A and B were made up in distilled water. Each contained a combination of sugars and sugar derivatives, that according to the experimentally established R_g values (the average of 5 measurements), gave the least overlapping of spots (see Table I).

TABLE I

Standard A	*R_g value*	*Standard B*	*R_g value*
D-Glucuronic acid	9 ± 2.0	D(+) Glucosamine · HCl	59 ± 3.4
D(+)Galactosamine · HCl	43 ± 4.0	D(+)Glucose	100
D(+)Galactose	85 ± 4.0	D(+)Mannose	126 ± 1.7
N-Acetyl-D-galactosamine	139 ± 2.6	N-Acetyl-D-glucosamine	150 ± 3.4
D(−)Ribose	185 ± 2.9	D(+)Fucose	175 ± 4.1
D-Glucuronolactone	249 ± 12.0	D-Glucuronolactone	249 ± 12.0

All sugars and derivatives were prepared in 1 μg/μl concentrations. Glucuronolactone, which was less sensitive than all other compounds used, was prepared at the 5 μg/μl concentration in standard solution B.

Sheets of 41.5 × 15 cm Whatman No. 1 paper were used for chromatography. The origin was 7 cm from the short side of the paper. Eight spots were placed 1.5 cm apart, the first 4 spots received 1, 2, 4 and 6 μl of standard A and the next four spots the same volumes of standard B. Descending chromatography was carried out in *n*-butanol–pyridine–water (10 : 3 : 3, v/v) for 25 h at room temperature. After this period of time they were left to dry in the fume hood until only a faint odour of *n*-butanol remained (approximately 4 h).

The sugar and sugar derivatives were then visualized by using one of the following reagents:

(a) The silver nitrate-alkaline reagent according to TREVELYAN *et al.*[10].

(b) 2% *p*-anisidine · HCl in methanol spray[6,7], followed by heating at 100° until spots were developed (2–10 min).

(c) The hexosamine (ELSON–MORGAN) reagent as outlined by SMITH[5]. After the second dipping the spots developed to full intensity within 2 min, then faded.

(d) (e) (f) (g) The aniline–diphenylamine (ADPA), naphthoresorcinol (NR), dinitrosalicylic acid (DNSA) and benzidine reagents were all applied according to SMITH[5].

(h) The aniline hydrogen phthalate (AHPh) reagent was made up and applied according to WILSON[11]. The chromatogram was air dried and then heated at 105° for 2 min to develop the spots.

(i) The periodate–acetylacetone (PAA) reagent was used as described by WEISS AND SMITH[12].

(j) The periodate–permanganate reagent was sprayed as described by LEMIEUX AND BAUER[8] and the chromatogram left to develop for 20 to 30 min at room temperature.

(k) (l) Both the 2,3,5-triphenyl tetrazolium chloride (TTC) and 3,3'-dianisole

TABLE II

DETECTION OF SUGARS AND SUGAR DERIVATIVES ON WHATMAN No. 1 PAPER

Compound	$AgNO_3$–alkaline		*p*-Anisidine·HCl		Elson–Morgan		ADPA		NR		DNSA		Benzidine		AHPh		PAA		PP		TTC		BT	
	colour	µg[a]	colour	µg	colour	µg	colour	µg	colour	µg	colour	µg	colour	µg	colour	µg	colour[b]	µg	colour	µg	colour	µg	colour	µg
D(+)Glucose	bn[d]	1	gr-bn	1	b-g	1	b-g	4	—	—	bn	4	bn	1	bn	1	y-gr	1	y	1	r	1	b-v	1
D(+)Galactose	bn	1	gr-bn	1	b-g	6	b-g	6	—	—	bn	6	bn	2	bn	2	y-gr	1	y	2	r	1	b-v	1
D(+)Mannose	bn	1	gr-bn	2	—[c]	—	b-g	6	—	—	bn	6	bn	1	bn	2	y-gr	1	y	1	r	1	b-v	1
D(+)Fucose	bn	1	gr-bn	2	b-g	6	—	—	—	—	bn	6	—	—	bn	2	—	—	y	1	r	1	b-v	1
D(−)Ribose	bn	2	r	1	b-g	6	—	—	—	—	bn	6	bn	2	r	2	y-gr	6	y	4	r	1	b-v	1
D-Glucuronic acid	d-bn	2	o-bn	1	gr-b	2	g	1	b	1	bn	4	g-bn	1	o-bn	1	y-gr	4	y	1	r	1	b-v	1
D-Glucuronolactone	bn	5	bn	20	—	—	b-g	20	b	5	—	—	g-bn	20	o-bn	20	—	—	y	5	r	2	b-v	2
D(+)Glucosamine·HCl	bn	2	gr-bn	6	r	1	—	—	—	—	—	—	l-bn	1	bn	2	y-gr	1	y	2	r	1	b-v	1
D(+)Galactosamine·HCl	bn	2	gr-bn	6	r	1	—	—	—	—	—	—	l-bn	1	bn	2	y-gr	1	y	2	r	1	b-v	1
N-Acetyl-D-Glucosamine	bn	4	gr-bn	4	p	1	—	—	—	—	—	—	—	—	bn	4	y-gr	1	y	2	r	1	b-v	1
N-Acetyl-D-Galactosamine	bn	4	gr-bn	4	p	1	—	—	—	—	—	—	—	—	bn	4	y-gr	1	y	2	r	1	b-v	1

[a] Sensitivity in micrograms.

[b] Colour as seen under U.V.

[c] — = Not detected at levels applied.

[d] Abbreviations used: ADPA = aniline-diphenylamine, NR = naphthoresorcinol, DNSA = dinitrosalicylic acid, AHPh = aniline hydrogen phthalate, PAA = periodate-acetylacetone, PP = periodate-permanganate, TTC = 2,3,5, triphenyl tetrazolium chloride, BT = blue tetrazolium; b = blue, bn = brown, d = dark, g = grey, gr = green, l = light, o = orange, r = red, p = purple, y = yellow.

bis-4,4(3,4-diphenyl)-tetrazolium chloride (or blue tetrazolium = BT) were used according to SZABADOS *et al.*[13] but the heating step was eliminated to avoid excessive background colour. Instead the chromatogram was left to develop for 24 h in the dark.

RESULTS AND DISCUSSION

The relative sensitivity of various reagents for the detection of sugars and sugar derivatives are summarized in Table II. Although in our work the detection of a minimum of 1 μg of an individual sugar was sufficient, the 1 μg levels given in Table II were not necessarily the limit of detection.

The silver nitrate–alkaline test gave distinct brown spots on a light brown background at low levels of all compounds, except N-acetyl-aminosugars and glucuronolactone. The latter formed an elongated spot on Whatman No. 1 paper, which may partially account for its apparent insensitivity in all tests. A similar elongation was reported by WILSON[11] for galacturonolactone using Whatman No. 1 and the same solvent system. The test was not specific for an individual sugar or chemically related group of sugars or sugar derivatives but was one of the more sensitive reagents for detection of well separated sugars. Because of its sensitivity and acceptance by many carbohydrate chemists, this method was compared with other methods.

The dinitrosalicylic acid reagent was insensitive. This may be partly due to the limited temperature range for colour development. Slight overheating of the chromatogram changed the yellow background to brown and thereby masked the similar colour of the spots. The aminosugars, N-acetyl-aminosugars and glucuronolactone did not react at the levels applied. Besides the low sensitivity for those sugars that did react, the test was non-specific.

The *p*-anisidine · HCl was very sensitive except with aminosugars and glucuronolactone. Most compounds gave a green-brown colour with this spray reagent, but glucuronic acid, glucuronolactone and ribose gave an orange-brown, brown and red colour respectively. VEIGA AND CHANDELIER[9] reported other colours for the same compounds under slightly different conditions. This general reagent for sugars gave specific colours in the case of the uronic acids and the single pentose used in this experiment.

The ELSON–MORGAN reagent was more sensitive than silver nitrate–alkaline for aminosugars and N-acetyl-aminosugars. Glucuronolactone and mannose could not be detected at the levels applied, but glucose, galactose, fucose and ribose gave blue-green spots at 6 μg or less. Aminosugars and N-acetyl-aminosugars could be distinguished from other sugars by their red and purple colouration respectively. Glucuronic acid gave a reddish spot after the first heating step but changed to greenish-blue after the second heating. The spots will fade unless heated at a specific stage during the removal of hydrochloric acid. Excess hydrochloric acid resulted in a brownish background upon heating, which masked the red and purple spots. Therefore, judgement was required when to start heating the chromatographic paper. The sensitivity and specificity of this reagent for aminosugars and N-acetyl-aminosugars made it especially useful as a companion to the silver nitrate–alkaline reagent.

It has been reported earlier[5], that the aniline–diphenylamine reagent was difficult to apply, but was useful for colour differentiation. However, it was found to be insensitive to most sugars at the levels considered in this work. The blue-grey back-

ground might have been responsible, since it tended to blend with the similar colours and intensities of the individual sugar spots. Glucuronic acid was the only compound detected at the 1 μg level. SMITH[5] reported the detection of fucose and ribose, but in our laboratory these two sugars failed to show at the levels applied.

The naphthoresorcinol reagent, specific for uronic acids, was more sensitive for glucuronic acid (1 μg) than for glucuronolactone (5 μg). Both of these sugar derivatives gave a very distinct bluish spot on a pink background. Neutral sugars and hexosamines would not react at the levels applied. There is considerable difference in the specific gravities of the chemicals used in this reagent mixture. Care was taken to insure homogeneity, or the test was no longer specific and other sugars also gave a bluish spot.

Aniline hydrogen phthalate had a sensitivity similar to silver nitrate–alkaline for aminosugars, N-acetyl-aminosugars and glucose. Other compounds except uronic acids, were detected at the 2 μg level. Of the uronic acids, glucuronic acid was detected at the 1 μg level, but glucuronolactone was not detected below the 20 μg level. All sugars or sugar derivatives gave a brown coloured spot on the paper chromatogram, except ribose and uronic acids, which were red and orange-brown respectively. Thus in addition to a sensitivity approaching that of silver nitrate–alkaline, aniline hydrogen phthalate reagent gave specific colours for the uronic acids and ribose.

The sensitivity of the benzidine reagent was of the same order as silver nitrate–alkaline for most compounds (1–2 μg). Fucose and N-acetyl-aminosugars were not detected when treated with this reagent.

The recent method (PAA) by WEISS AND SMITH[12] could be used for the detection of most sugars and sugar derivatives in the range of 1 to 6 μg. The spots showed up well under U.V., but the visible yellow colour was rather faint. Fucose and glucuronolactone were not detected using as high as 6 and 30 μg respectively. This method detected hexosamines at the same level (1 μg) as in the ELSON–MORGAN test.

The periodate–permanganate reagent reacted with low concentrations of all compounds, especially the more difficultly detected amino- and N-acetyl-aminosugars. Except for ribose, the reagent was as sensitive as the silver nitrate–alkaline. No colour differentiation was observed.

The data in Table II show, that TTC and BT were sensitive for all sugars and sugar derivatives at the 1 μg level with the notable exception of glucuronolactone, which was detected at the 2 μg level. Both tests, however, were considerably more sensitive for glucuronolactone than any other method used. Although most sugars could be detected after 1 h development in the dark, mannose and glucuronolactone did not reach full intensity before 24 h. All sugars and sugar derivatives used in this study gave a red colour upon development.

Table II illustrates that if N-acetyl-aminosugars or glucuronolactone were present in a hydrolysate, the number of methods for detection at the 1–2 μg level was limited. Hexosamines may be detected at the 1 μg level by ELSON-MORGAN, PAA, TTC and BT reagents. Glucuronolactone was relatively insensitive, although it was detected below the 6 μg level by 5 reagents. The results seem to indicate that redox reactions, such as the ones involving silver nitrate–alkaline[14], periodate–permanganate[8], TTC and BT[15] are more sensitive, but less specific for individual sugars,

Reactions involving at one stage or another a condensation such as the ELSON–MORGAN[16], NR[17] and those using primary aromatic amines[18] appear to be less sensitive, but more specific.

CONCLUSIONS

For the overall detection of sugars and sugar derivatives several reagents may be employed, such as silver nitrate–alkaline, periodate–permanganate, and the triaryl tetrazolium salts. Of these reagents 2,3,5-triphenyl tetrazolium chloride and Blue tetrazolium were the most sensitive. For a combination of sensitivity and specificity the ELSON–MORGAN and naphthoresorcinol tests were the most applicable to amino sugars and uronic acids respectively, whereas the aniline hydrogen phthalate differentiated best between hexoses, uronic acids and the one pentose.

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