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Chromatographic separation of 1-phenyl-3-methyl-5-pyrazolone-derivatized neutral, acidic and basic aldoses

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Abstract

Analysis, by HPLC, of reducing monosaccharides as their 1-phenyl-3-methyl-5-pyrazolone derivatives is attractive owing to its sensitivity of detection and the generation of single derivatives of each aldose molecule [Honda et al., *Anal. Biochem.* 180 (1989) 351]. The present studies establish conditions for reversed-phase chromatographic analyses of hydrolysates containing neutral, basic and acidic reducing monosaccharides. In particular, glucuronic and galacturonic acids, glucosamine and galactosamine are separated completely both from one another and from the aldoses of glycoproteins commonly found. Analyses of blank hydrolysates provide the baseline for background amounts of these carbohydrates and analyses of a variety of glycoproteins illustrate the effectiveness of these separations. Elution times were established for derivatives of all tetroses, pentoses and hexoses, as well as a variety of deoxy and phosphorylated aldoses.

1. Introduction

The state of glycosylation can have a major influence on the structure and biological activity of proteins [1,2]. The carbohydrate moieties participate in such functions as compartmentalization, transport and excretion, cell–cell communication and protein folding. The importance of proteoglycans is also increasingly apparent [3]. Analytical examination of the sugar content of peptides and proteins is therefore a fundamental requirement for research on and production of glycoproteins.

Typically, sugar compositional analyses are performed by ion-exchange chromatography of hydrolyzed sugars at high pH and with direct detection using electrochemical detectors (e.g. [4]) or through post-column reactions [5,6], or alternately by reversed-phase chromatographic analyses of variously derivatized sugars, derivatized e.g. with reagents such as dabsyl- and fluorenylmethoxycarbonyl hydrazines [7,8], phenylisothiocyanate [9] or benzoylation reagents [10]. The ion-exchange techniques require more specialized equipment than the pre-column derivatization methods, while the latter in many cases yield multiple peaks for each sugar [11,12]. Honda et al. [12] developed 1-phenyl-3-methyl-5-pyrazolone (PMP) as a pre-column derivatization reagent which yields highly absorbent single derivatives in good yield. The methodology has found use in other laboratories [13–15], but the

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generation and separation of PMP derivatives of the commonly found free-amino sugars and uronic acids has not yet been described.

We describe here a chromatographic analysis of the neutral, acidic and basic reducing sugars which are found in hydrolysates of glycoproteins and proteoglycans.

2. Experimental

2.1. Materials

Ovalbumin was obtained from Pharmacia Biotech (Piscataway, NJ, USA), phósvitin and actin from Sigma (St. Louis, MO, USA) and a recombinant angiotensin-converting enzyme mutant was a gift of Dr. M.R.W. Ehlers. The various sugars were from Sigma, except for galactose (Fisher Scientific, Pittsburgh, PA, USA) and ribose (Nutritional Biochemical, Cleveland, OH, USA). PMP was from Aldrich (Milwaukee, WI, USA) and was recrystallized from methanol [13] (m.p. 127–128.5°C) before use.

2.2. Hydrolysis of glycoproteins

The samples (100–1000 pmol) were dried into 50×6 mm glass test tubes which had been cleaned by pyrolysis in a muffle furnace at 500°C for 18 h. Trifluoroacetic acid (200 μl, 2 M) was added into each tube and the tubes placed in a hydrolysis vial (PicoTag; Millipore, Milford, MA, USA), briefly evacuated, sealed and placed at 110°C for the required time. Hydrolysates were dried under vacuum in 2-ml conical glass tubes, ready for derivatization.

2.3. Derivatization with PMP

Dry samples were derivatized [12] with 10 μl PMP (0.5 M in methanol) and 10 μl 0.3 M sodium hydroxide at 70°C for 30 min. The derivatives were neutralized with 3.5 μl of 1 M HCl, dried, redissolved in 50 μl water and excess reagent extracted twice with 200 μl chloroform. The aqueous layer was analyzed directly by HPLC.

2.4. Chromatography

Chromatographic conditions were generally as follows: column, NovaPak 30×0.39 cm (Millipore); temperature, 26°C; solvent A, 0.4% triethylamine (pH 4.86 with phosphoric acid) + 10% acetonitrile in water; solvent B, acetonitrile–water (60:40); gradient, 10–14% B in 9 min, 14–64% B in a further 21 min at 1 ml/min flow-rate. The eluate was monitored at 254 nm.

3. Results and discussion

3.1. Hydrolysis and derivatization

Hydrolysis of simple carbohydrate chains was essentially complete after 2 h of hydrolysis at 110°C, with only a slight increase in the yields of amino sugars at 4 h. The hydrolysis of ATP/ADP to ribose was slower and required a minimum of 4 h hydrolysis.

Derivatization by the procedure of Honda et al. [12] proceeded smoothly, when care was taken to ensure that the aqueous phase had been fully neutralized prior to extraction with chloroform. At high pH the extractions were inefficient and significant amounts ($>50\%$) of the PMP-sugars were also extracted. Attempts to use ethyl acetate [16] as extractant were unsuccessful owing to excessive losses of the PMP-sugars.

The derivatization of the uronic acids and the free amino sugars also proceeded well with single peaks appearing during HPLC analyses of the reaction mixtures. The peak areas (color yields) for equal amounts of most PMP-sugars were within 10% of their average value, with glucuronate deviating the most with a color yield which was 27% low (Table 1). The derivatives are stable for a day at room temperature and in solution, which allowed convenient overnight HPLC analyses. The most labile derivatives in the group, that decrease in apparent yield over periods of days, are those of glucose, glucosamine, glucuronic acid and galactosamine.

Table 1
Relative color yields of PMP-sugars, reported as average ± standard deviation

PMP-sugar	Relative color yield fresh standards ($n = 15$)	Relative color yield decayed standards ($n = 5$)
Mannose	1.10 ± 0.04	0.98 ± 0.17
Glucosamine	0.89 ± 0.16	0.41 ± 0.18
Lyxose	1.07 ± 0.03	1.14 ± 0.10
Ribose	0.95 ± 0.03	1.07 ± 0.11
Galactosamine	1.21 ± 0.17	0.71 ± 0.22
Glucuronate	0.73 ± 0.04	0.57 ± 0.12
Galacturonate	1.03 ± 0.06	1.12 ± 0.23
Glucose	0.86 ± 0.07	0.71 ± 0.09
Galactose	0.98 ± 0.05	0.90 ± 0.10
Xylose	1.08 ± 0.10	1.09 ± 0.10
Fucose	0.94 ± 0.08	0.99 ± 0.10

3.2. Separation of standard PMP-sugars

Initial experiments showed that a large, broad reagent peak eluted early during chromatography and could be moved relative to the PMP-sugars by changes in slope of the acetonitrile gradient or by varying the initial acetonitrile concentration. Higher acetonitrile concentrations eluted the reagent peak in very close proximity to the PMP-sugars. The chromatographic system was therefore designed to provide a large separation of reagent and the earliest eluting PMP-sugar, PMP-mannose. A very flat initial gradient proved essential to this aim. A second linear gradient provided separation of most of the other neutral compounds of interest. A separation of the common neutral, acidic and basic PMP-sugars is presented in Fig. 1. The broad reagent peak separates very well from all the PMP-sugars, and good separation was achieved for derivatives of mannose, glucosamine, lyxose, ribose, galactosamine, glucuronic acid, galacturonic acid, glucose, galactose, xylose and fucose, all of which elute as very sharp peaks.

Since good yields of PMP-glucosamine and -galactosamine were obtained using both standard compounds and glycoprotein hydrolysates, the separation was designed for hydrolysis mixtures which would obviously contain free amino sugars and not N-acetyl amino sugars, and which

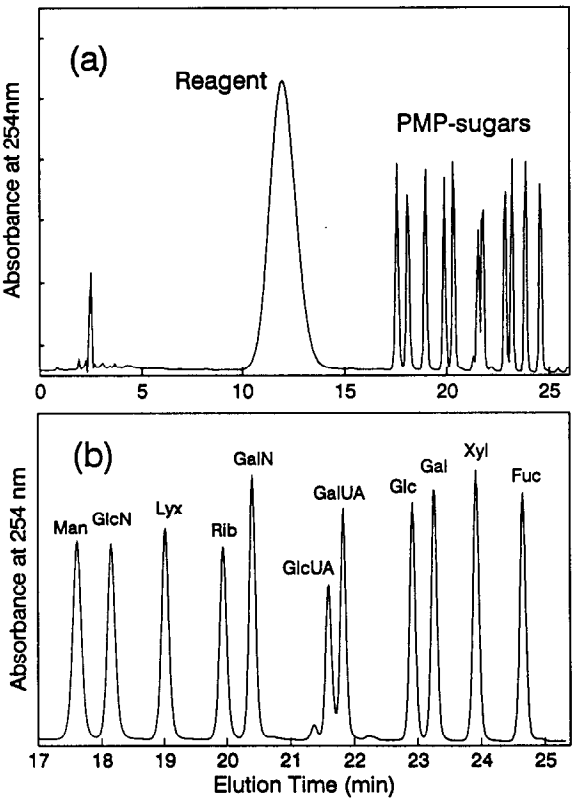

Fig. 1. Chromatography of PMP derivatives of common reducing monosaccharides. (a) Separation of the remaining reagent (broad peak) from the PMP derivatives (sharp peaks). (b) An expanded region of the chromatogram in (a): the PMP derivatives of the common neutral, basic and acidic reducing monosaccharides. Man = Mannose; GlcN = glucosamine; Lyx = lyxose; Rib = ribose; GalN = galactosamine; GlcUA = glucuronic acid; GalUA = galacturonic acid; Glc = glucose; Gal = galactose; Xyl = xylose; Fuc = fucose.

are more amenable to chromatographic manipulation. Fig. 2 shows the variation of elution times of the uronic acids and amino sugars as a function of pH. Values of pH from 4.5 to 5.3 allowed PMP-glucosamine to elute between PMP-mannose and PMP-lyxose, while PMP-galactosamine eluted between PMP-ribose and PMP-glucuronic acid. Higher pH values altered the relative elution of the charged sugars much more than that of the others. The neutral sugars show highest resolution in the tested range at pH 6.9, although the amino sugars then elute in inconvenient positions. At this pH PMP-arabin-

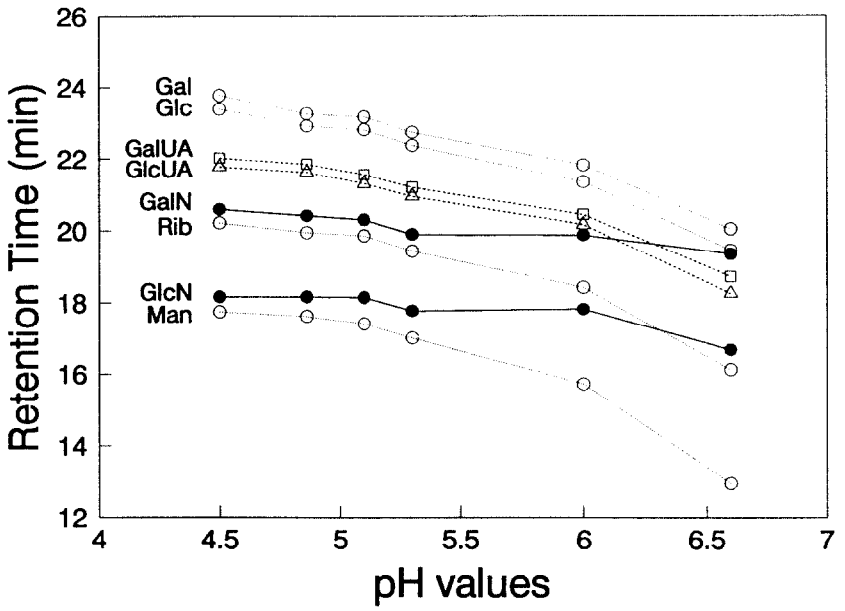

Fig. 2. Chromatographic elution times of PMP-sugars at different pH values of the eluent.

ose separates from PMP-xylose, eluting slightly later.

3.3. Separation of less common PMP-aldoses

The other hexoses, pentoses, tetroses, glyceraldehyde and some deoxy and phosphorylated sugars were derivatized and chromatographed as above. Many separated from the common sugars and their elution positions are indicated in Fig. 3. The deoxy sugars eluted later than their parent compounds, as expected for their higher hydrophobicity, while the phospho sugars eluted earlier, in accord with their higher hydrophilicity.

The elution order of the various enantiomorphic pairs of aldotetroses, -pentoses and -hexoses appears to depend on the orientation of the hydroxyl groups in positions 2 and 3. A *cis*-orientation correlates with early elution and a *trans*-orientation with late elution. This causes the slowest eluting of the 2,3-*cis* enantiomorphs of tetroses, pentoses and hexoses —PMP-erythrose— to elute before the earliest of the *trans* enantiomorphs, PMP-idose.

In addition hydrolysates of heparin and alginic acid were analyzed to examine the potential of the method for proteoglycans. In the former case a large variety of fragments were seen, presumably sulfated products, but glucuronic acid and glucosamine were clearly identified. The expected products from hydrolysis of alginic acid are mannuronic and guluronic acids and two major peaks were found to elute between the positions of mannose and gulose. Ascorbate reacted with PMP and yielded two products which eluted very early, preceding and superimposed on the reagent peak.

3.4. Adventitious contamination by sugars

The background contribution of sugars arising during the hydrolysis process could become a problem when sensitive analytical methodologies such as the PMP method are used to estimate amounts of sugar in relatively small amounts of glycoprotein, or when a protein is examined for a potential short-chain carbohydrate modification. This contamination may be serious and is inherent in the hydrolysis acid, as well as being introduced from the environment. Table 2 records the amounts of the neutral aldoses found

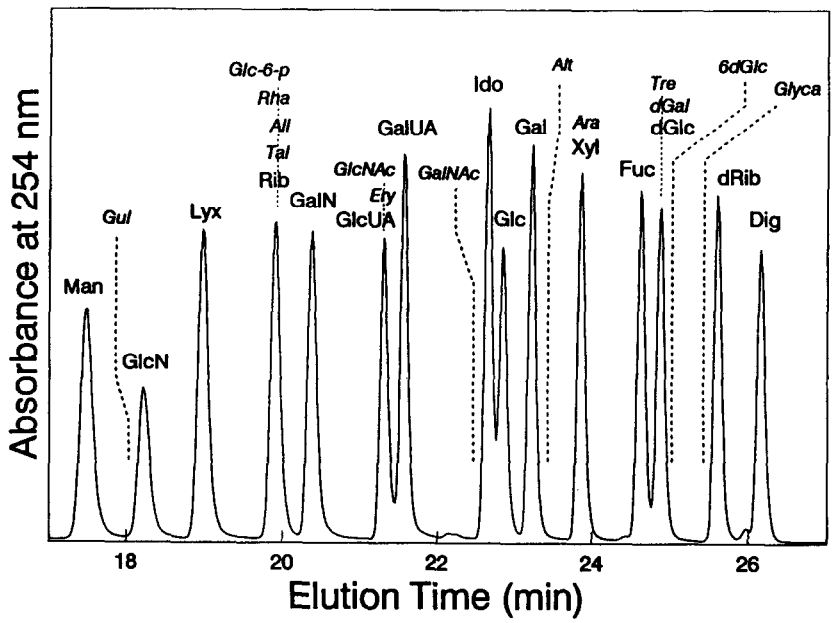

Fig. 3. Chromatography of PMP-sugars. Fifteen well resolved compounds are shown, with the elution positions of other derivatives indicated. Gul = Gulose; Glc-6-p = glucose-6-phosphate; Rha = rhamnose; All = Allose; Tal = Tallose; GlcNAc = N-acetylglucosamine; Ery = erythrose; GalNAc = N-acetylgalactosamine; Ido = idose; Alt = Altrose; Ara = arabinose; Tre = threose; d = deoxy; Glyca = glyceraldehyde; Dig = digitoxose; other abbreviations as in Fig. 1.

in hydrolysis blanks done singly over a period of time. Glucose and xylose are particularly problematic and contents of unknown samples should therefore be interpreted with caution when <100 pmol of protein is analyzed. Reaction blanks, without hydrolysates, give very clean chromatograms with values of <2 pmol on average for everything except for glucose, for which it is <4 pmol.

The amounts of amino sugars and uronic acids in the hydrolysis blanks were as negligible as in the reaction blanks.

Table 2
Analysis of sugars in hydrolysis blanks

Sugar	Average amount ± S.D. (pmol), $n = 27$	Range (pmol)
Mannose	13.6 ± 12.5	0–53.2
Glucose	74.0 ± 46.4	12.8–200.8
Galactose	9.5 ± 5.3	3.1–21.8
Xylose	43.7 ± 34.7	0–136.1
Fucose	4.0 ± 4.8	0–17.1

Blanks were made by adding 200 μl 2 *M* trifluoroacetic acid into clean test tubes inside hydrolysis vials and hydrolyzing together with batches of samples. Derivatization with PMP was done as described under Experimental and the derivatives were analyzed by reversed-phase HPLC

3.5. Applications to glycoproteins

A variety of glycoproteins were analyzed by the chromatographic methodology outlined above. The results are summarized in Table 3. Identification and quantitation correlated well with the literature values for ovalbumin and phosvitin. Actin preparations contain a mole of either ATP or ADP which yield ribose on acid hydrolysis. The analysis of a recombinant mutant of angiotensin converting enzyme demonstrates the ease with which the variety of carbohydrate residues in such a heavily glycosylated protein can be determined.

The accurate analysis of amounts of less than 100 pmol glycoprotein becomes difficult owing to

Table 3
Analysis of the sugar residues of glycoproteins by chromatography of PMP derivatives

Sugar	ACE[a] mutant	Phosvitin	Ovalbumin	Actin
Mannose	14.8	3.45 (3 [18])	5.57 (4–6 [19], 6 [20])	0.15
Glucosamine	23.3	5.23 (5 [18])	3.79 (2–5 [19], 3 [20])	0.00
Galactosamine	6.5	0.10	0.03	0.04
Glucose	1.5	0.65	0.30	0.99
Galactose	28.2	3.46 (3 [18])	0.36 (0–1 [19])	0.10
Xylose	0.5	0.36	0.16	0.16
Fucose	5.9	0.12	0.13	0.03
Glucuronic acid	0.0	0.03	0.04	0.04
Galacturonic acid	0.0	0.01	0.03	0.01
Ribose	0.4	0.17	0.07	0.44 (1)
Amount hydrolyzed (pmol)	95.2	543	1116	486

Proteins were hydrolyzed with 2 *M* trifluoroacetic acid for 4 h at 110°C. Values are reported as molar ratios recovered. Literature values are in parentheses.
[a] Angiotensin converting enzyme.

the prevalent background of especially glucose, seen both in blank samples and also as an additional background in protein preparations. This has also been noted in analyses done by anion-exchange chromatography with electrochemical detection [4].

4. Conclusions

The determination of the monosaccharide content of glycoproteins provides basic information, much as amino acid analyses provide a characteristic property of a protein. We have established a robust reversed-phase HPLC separation of stable derivatives of reducing sugars which allows convenient analysis of carbohydrate compositions using conventional HPLC equipment. It has already proved useful in studying the glycosylation state of angiotensin-converting enzyme [14]. Analysis of qualitatively unknown samples may occasionally provide derivatives other than the common ones. A recent example is the discovery of gulose in an algal glycoprotein [17]. The use of the high-resolution separation, which is very robust in the pH range from 4.9 to 5.3, allows such derivatives to be recognized with ease. The use of a second eluent system, with a different pH (such as 6.9) will clearly differentiate sugars such as gulose or arabinose from the common sugars.

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