Note

Gas chromatographic determination of glycoprotein amino sugars as O-pentafluorobenzyloxime acetates

PIER ANTONIO BIONDI*, FRANCESCA MANCA, ARMANDO NEGRI, GABRIELLA TEDESCHI and CAMILLO SECCHI

Istituto di Fisiologia Veterinaria e Biochimica, Via Celoria 10, 20133 Milan (Italy)

(First received August 29th, 1988; revised manuscript received January 9th, 1989)

We recently introduced a method for the gas chromatographic (GC) determination of glycoprotein neutral monosaccharides[1]. The procedure requires derivatization with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBOA) followed by acetylation to give stable pentafluorobenzyloxime acetates (Ac-PFBO). The separation of the Ac-PFBO derivatives was performed on fused-silica capillary columns coated with a polar cyanopropylsiloxane phase. However, we could not achieve a reliable simultaneous determination of the amino sugars present in glycoproteins, glucosamine (GlcN) and galactosamine (GalN). Adsorption of amino sugars on both glass and fused-silica capillary columns is a well known problem[2-4].

The aim of this work was to establish improved conditions that allow the method to be extended to the determination of both neutral and amino sugars. In addition, the reproducibility of the method was increased by using 3-O-methylglucose (OMeGlc) instead of *meso*-inositol as an internal standard for the final quantitative analysis. The glycidic content of standard glycoproteins is reported as an application of the improved procedure.

EXPERIMENTAL

Materials

PFBOA was supplied by Janssen (Beerse, Belgium), neutral and amino sugars by Fluka (Buchs, Switzerland) and standard glycoproteins by Sigma (St. Louis, MO, U.S.A.).

Apparatus

A DANI Model 3600 B gas chromatograph equipped with a flame ionization detector and adapted for capillary columns was used. Electron-impact mass spectrometric (MS) analysis was carried out with a Finnigan-MAT 1020 instrument at an ionizing voltage of 70 eV.

Chromatographic conditions

The separations were performed on a fused-silica column (6 m × 0.32 mm I.D.) wall-coated (0.2 μm) with CP-Sil 88 (Chrompack, Middelburg, The Netherlands). The

following temperatures were applied: column, 140°C for 4 min, then 10°C/min to 240°C and held for 10 min; injector, 240°C; and detector, 250°C. Splitless injection was used (splitless time 40 s). The gas flow-rates were as follows: carrier gas (helium), 3 ml/min; hydrogen, 25 ml/min; and air, 350 ml/min. The carrier gas was purified by Gas-clean moisture and oxygen filters (Chrompack).

Derivatization

The procedure is only slightly different from that reported previously[1]. Aliquots of neutral and amino monosaccharides and of OMeGlc were withdrawn from their standard aqueous solutions (10 or 1 mM) and dried under vacuum over potassium hydroxide pellets. The reaction and extraction steps were carried out in PTFE-lined screw-capped vials. A 0.2-ml volume of pyridine containing 10 mg of PFBOA was added to the dried monosaccharides and the mixtures were maintained in an ultrasonic bath for 5 min. The tubes were then heated at 80°C for 20 min. After cooling, acetic anhydride (0.4 ml) was added and the samples were heated again for 20 min at 80°C. The reaction mixtures were evaporated nearly to dryness under a stream of nitrogen and the residues were dissolved in dichloromethane (3 ml) and washed with ice-cold 0.1 M hydrochloric acid (2 ml) and water (2 × 2 ml). The organic phases were filtered over anhydrous sodium sulphate and evaporated to dryness under a stream of nitrogen. The residues were dissolved in ethyl acetate (0.1 ml) and aliquots (1 μl) were analysed by GC.

Glycoprotein hydrolysis and determination were performed according to the previous protocol[1] with the exception of the use of OMeGlc as an internal standard. Calibration graphs were constructed in the ranges 5–500 and 10–1000 nmol for neutral and amino sugars, respectively. OMeGlc (50 nmol) was always added.

RESULTS

A typical chromatographic profile obtained from a sample containing neutral and amino sugars and OMeGlc as internal standard is shown in Fig. 1. All unknown peaks resulting from the reagents, solvents and occasional impurities are eluted before
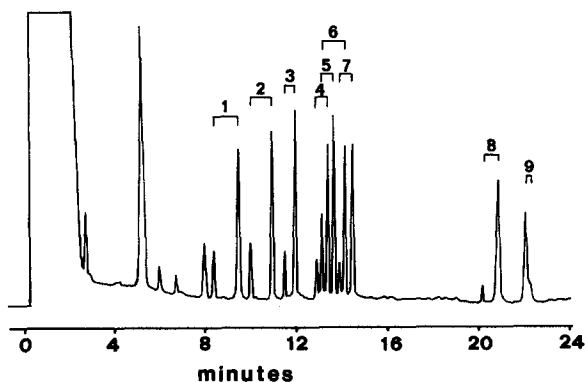

Fig. 1. Typical GC profile corresponding to a standard sample of neutral and amino sugars analysed according to the conditions described under Experimental. Peaks: 1 = fucose; 2 = arabinose; 3 = xylose; 4 = O-methylglucose; 5 = mannose; 6 = galactose; 7 = glucose; 8 = glucosamine; 9 = galactosamine.

fucose isomers. No other interferent peak hinders the analysis of the sugar derivatives, eluted with longer retention time owing to their higher molecular weight. In the profile, one of the two isomers of Ac-PFBO-GalN was present only as a shoulder on the predominant peak; nevertheless, its determination was reliable. In fact, the plot of the ratios between the height of the peak of Ac-PFBO-GalN and that of the predominant peak of Ac-PFBO-OMeGlc *versus* the amount of hexosamine was linear. The same linear relationship was obtained for the predominant isomers of all other monosaccharides. The parameters of the calibration graph are reported in Table I. The minimum detectable amount of GlcN and GalN was about 4 nmol, corresponding to 40 pmol injected.

The identity of the hexosamine derivatives was verified by MS. Mass spectra were recorded for both the minor and the predominant peaks of Ac-PFBO-GlcN and at the beginning and end of the Ac-PFBO-GalN peak. The portions of the spectra at m/z values higher than 300 are shown in Fig. 2 (the base peak was at 115 m/z for both derivatives and therefore it does not appear). The background noise is due to the high sensitivity used to show significant peaks. The structure and one of the characteristic fragmentations of hexosamine derivatives are shown in Fig. 3. No differences were found between the spectra of the two isomers of each amino sugar derivative, indicating that MS analysis allows the identity of the derivative to be established but does not distinguish between *syn* and *anti* isomers, as already observed for neutral sugars.

In order to study the stability of GlcN and GalN to trifluoroacetic acid (TFA), the standard amino sugars were subjected to derivatization with and without previous TFA treatment. By comparing the resulting peak heights, the mean recoveries ($n = 3$) for 50 nmol were 78% (range 72–84%) and 92% (range 83–95%) for GlcN and GalN, respectively.

Sugar components of some standard glycoproteins were determined as an example of the application of the modified procedure; the results are reported in Table II and a typical chromatographic profile is shown in Fig. 4.

TABLE I

CALIBRATION PARAMETERS

Calibration according to the equation $R_h = a + b$(nmoles monosaccharide), where R_h is the ratio between the height of the major peak of each monosaccharide and the major peak of OMeGlc. The regression lines for calibrations are obtained with different amounts of monosaccharides and the same amount (50 nmol) of OMeGlc as internal standard. The values reported are the means of three determinations.

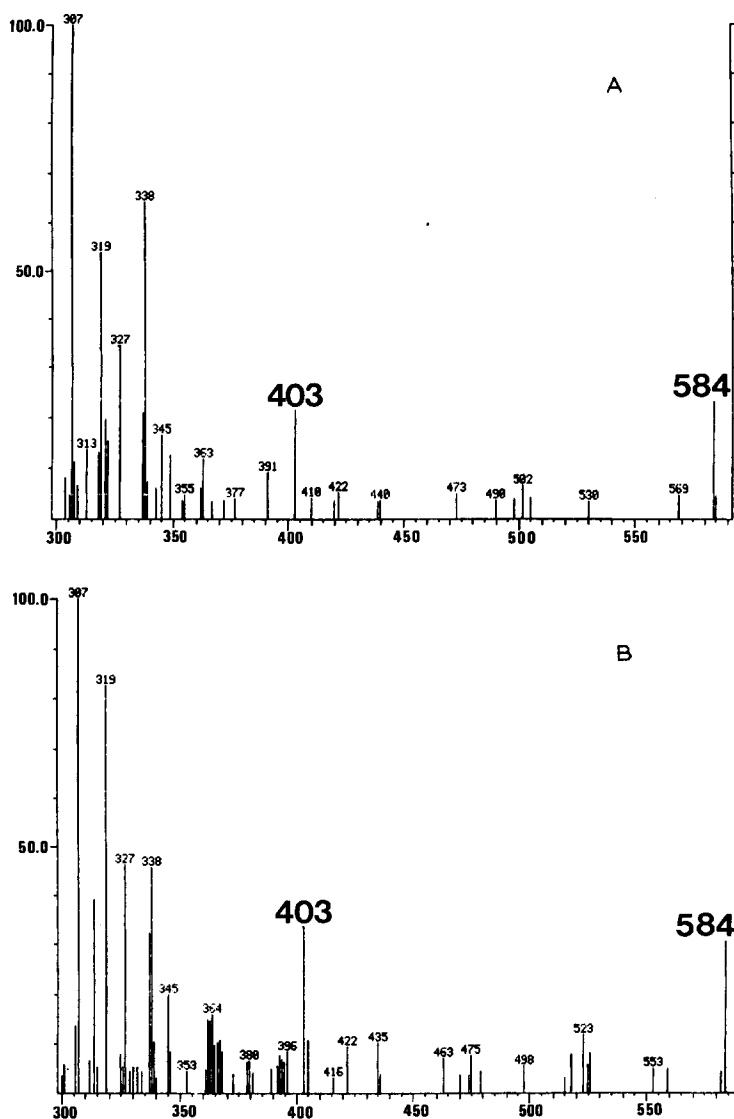| Monosaccharide	a (intercept)	b (slope)	r (correlation coefficient)
Fucose	0.049	0.019	0.998
Arabinose	0.028	0.021	0.999
Xylose	0.042	0.024	0.999
Mannose	−0.033	0.026	0.999
Galactose	−0.009	0.020	0.999
Glucose	−0.002	0.021	0.999
Glucosamine	0.051	0.0079	0.994
Galactosamine	0.020	0.0056	0.995

Fig. 2. Portions of mass spectra at m/z values higher than 300 of Ac-PFBO derivatives of (A) glucosamine and (B) galactosamine.

DISCUSSION

Previously, neutral sugars were separated as their Ac-PFBO derivatives on a 15-m capillary column coated with a cyanopropylsiloxane phase[1]. Under the reported conditions GlcN and GalN derivatives gave good and well resolved peaks only when brief linear high-temperature programmes (from 200 to 240°C at 15°C/min) were used. When the running time was increased by using lower initial temperatures,

TABLE II

CONTENTS OF ALDOSES AND HEXOSAMINES (%, w/w) IN SOME KNOWN GLYCOPROTEINS

Glycoprotein		Monosaccharide				
		Fucose	Mannose	Galactose	N-Acetylglucos-amine	N-Acetylgalactos-amine
Foetal calf serum	Found	–	2.6	4.3	6.7	1.1
asialofetuin	Ref. 5	–	2.9	4.2	5.9	1.0
Bovine thyroglobulin	Found	0.5	2.2	1.6	6.8	–
	Ref. 6	0.4	2.3	1.3	6.0	–
Bovine α_1-acid	Found	0.7	5.9	6.8	10.5	–
glycoprotein	Ref. 7	0.8	5.8	7.0	11.0	–
Ovalbumin	Found	–	2.1	0.2	1.1	–
	Ref. 8	–	2.4	0.15	1.3	–

the GlcN and GalN peaks broadened and their determination was unreliable. However, this applies only to new columns. In fact, with a column life as short as about 2 weeks the amino sugars peaks completely disappeared also using brief high-temperature programmes. This confirms the difficulties encountered by other workers with the GC analysis of amino sugars on fused-silica and especially glass capillary columns[2–4]. In our case, the use of PFBOA results in derivatives with high molecular weight and low volatility, which are more likely to be adsorbed than other derivatives commonly used in the GC analysis of monosaccharides (e.g., alditol acetates, aldonitrile acetates and O-methyl oxime acetates).

    In order to overcome this problem, we adjusted the length of the capillary column and increased the purity of the carrier gas. The column length was reduced from 15 to 6 m and helium was cleaned by filters retaining water and oxygen. The results were very satisfactory with regard to both efficiency and reproducibility of the amino sugar determination. The Ac-PFBO-GlcN and Ac-PFBO-GalN peaks were narrow and easily quantified at nanomole level also with the programme needed for the determination of all the monosaccharides. In fact, in spite of the use of a shorter column the separation of syn and anti isomers of the neutral sugars remained unaffected. Moreover, as the chromatographic behaviour of the hexosamine deriva-tives was independent of the column age, reliable routine analysis were assured for at least 4 months.

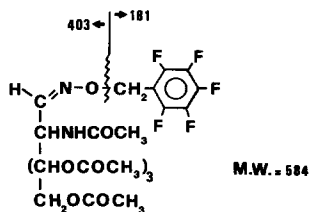   With regard to quantitative analysis, OMeGlc was preferred to the previously

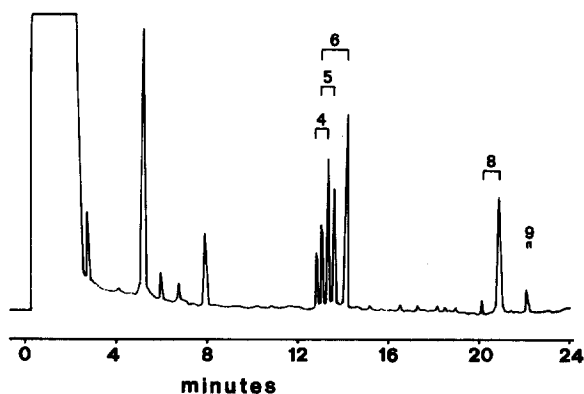Fig. 3. Structure and a characteristic fragmentation of Ac-PFBO derivatives of hexosamines.

Fig. 4. Typical GC profile corresponding to a sample of foetal calf serum asialofetuin analysed according to the described procedure. Peak numbers as in Fig. 1.

used *meso*-inositol as an internal standard because it is more similar chemically to monosaccharides. The correlation coefficients of the calibration graphs for neutral sugars reported in Table I are higher than those obtained with *meso*-inositol; this can be explained by the involvement of OMeGlc in both derivatization steps, whereas *meso*-inositol was involved only in the acetylation step. The other difference from the previous procedure is the smaller volumes of pyridine and acetic anhydride in the reaction mixtures (while the amount of PFBOA used is unchanged). The use of an ultrasonic bath, in fact, ensured good solubilization of the monosaccharides in a lower volume, while the final time-consuming evaporation step was speeded up.

ACKNOWLEDGEMENTS

REFERENCES

1 P. A. Biondi, F. Manca, A. Negri, C. Secchi and M. Montana, *J. Chromatogr.*, 411 (1987) 275.
2 J. R. Hudson, S. L. Morgan and A. Fox, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 5 (1982) 285.
3 A. Fox, S. L. Morgan, J. R. Hudson, Z. T. Zhu and P. Y. Lau, *J. Chromatogr.*, 256 (1983) 429.
4 J. R. Neeser and T. F. Schweizer, *Anal. Biochem.*, 142 (1984) 58.
5 J. R. Neeser, *Carbohydr. Res.*, 138 (1985) 189.
6 T. P. Mawhinney, M. S. Feather, G. J. Barbero and J. R. Martinez, *Anal. Biochem.*, 101 (1980) 291.
7 D. G. Pritchard and W. Niedermeier, *J. Chromatogr.*, 152 (1978) 487.
8 S. Honda and S. Suzuki, *Anal. Biochem.*, 142 (1984) 167.