A rapid micro-determination of neutral sugars and aminosugars in glycopeptides by thin-layer chromatography*

The key substances in the structural study of the sugar moiety of the glycoproteins are the glycopeptides obtained by the enzymatic digestion of the glycoproteins[1].

The first step of the structural investigation of glycopeptides is usually the qualitative and quantitative determination of the sugar components and the estimation of their molecular ratio.

The sensitivity of the available methods was not adequate for repeated analysis of small quantities of such substances. The paper** and thin layer chromatographic methods[2-4] need 10–100 μg of each individual sugar for a single determination.

Gas chromatographic analysis[5,6] is more sensitive, 1–10 μg sugar can be used for a determination. However, the apparatus is expensive and each sugar component gives two peaks at least, due to the separation of α and β anomers, complicating the quantitative evaluation of complex mixtures.

In the present work a quantitative thin layer chromatographic method is described for the separation and determination of neutral sugars and hexosamines, occurring usually in the hydrolysates of the glycopeptides. This method was recently applied to the study of the structure and composition of glycoproteins isolated from connective tissue (structural glycoproteins)[7-9].

Materials and methods

Hydrolysis of the samples. For the determination of hexoses, 0.1–0.03 mg of a glycopeptide with 10–40 % hexose content were hydrolysed in a sealed tube with 200 μl 2 N HCl at 100° for 1.5–3 h, depending on the nature of the glycopeptide[10]. The acid was evaporated in a desiccator *in vacuo* in the presence of KOH, and the residue was dissolved in 20 μl of water.

For the determination of hexosamines, similar quantities of glycopeptides were hydrolysed with 4 N HCl at 100° for 8 to 4 h according to the substance. The hydrolysate was treated as before.

Thin layer chromatography. Eastman Kodak 511 V sheets 10 × 10 cm impregnated with 0.2 M phosphate buffer at pH 6.8 were used[11]. From the solution prepared above, a volume containing about 1 μg of each sugar component was deposited as a 5–6 mm short line at 15 mm distance from the lower edge of the sheet. Six samples were applied on one sheet. Three microdrops of 0.1–0.5 μl were deposited side by side and the sheet was dried before the next aliquot of the sample was deposited. Propanol–ethyl acetate–water (5:1:1) mixture served as mobile phase yielding a good resolution of hexoses. For hexosamines, the solvent used was ethanol–25 % aqueous ammonia–water (85:0.5:14.5, by vol.). The sheets were developed by the ascending method.

Staining of the spots and quantitative evaluation. After drying, the developed sheets were dipped in a freshly prepared 1:1 mixture of a 1 % ethanolic triphenyltetrazolium chloride (TTC) solution and a 1 % methanolic sodium hydroxide solution. The sodium hydroxide should be dissolved just before use. The red color of the spots

* Supported by: Convention de Recherches DGRST, (Délégation Générale à la Recherche Scientifique et Technique), No. 66 00 441. Grant-NIH: NB-04693. Subventions du CNRS.

** See "Note added in proof" on p. 564.

was developed by heating the sheets at 90° in a water-saturated atmosphere for 15 min. After cutting the strips the color intensity of the spots was measured on a recording densitometer (Photovolt Corp., New York, N.Y.), and the area of the peak estimated by planimetry or by multiplying the height of the peak by its width at midheight.

Results and discussion

Determination of hexoses. Chromatograms and the corresponding densitometric recordings of the hydrolysate of the glycopeptides obtained by the digestion of structural glycoproteins[9] of the stroma of the rabbit cornea[7], porcine aorta[8] and a reference mixture of galactose, glucose and mannose, are illustrated in Fig. 1.

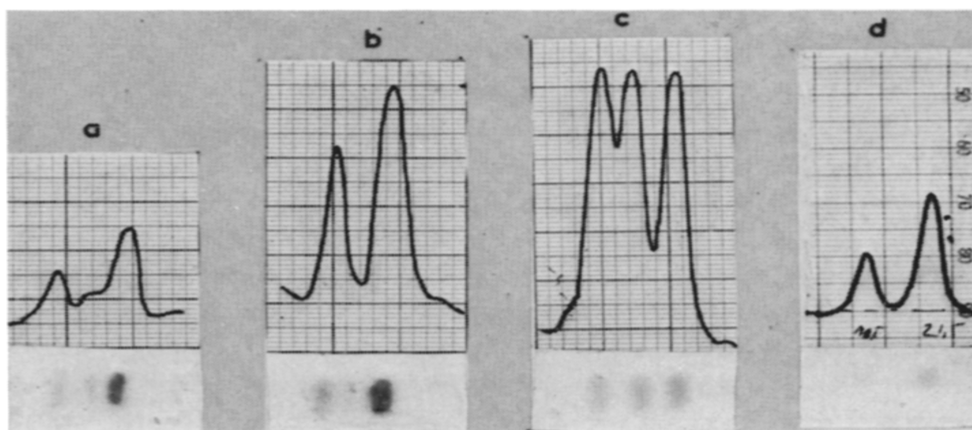

Fig. 1. Chromatograms of glycopeptides isolated from the structural glycoproteins of rabbit cornea (a), porcine aorta (b) and of standard mixtures of hexoses (2 μg galactose, 2 μg glucose, 2 μg mannose) (c) and hexosamines (0.25 μg galactosamine, 0.50 μg glucosamine) (d).

The red color given with the TTC by the three hexoses has an almost equal intensity in the range of 0.2–2.0 μg. In the same range, the galactose, glucose and mannose gave a linear relationship between the peak area and the concentration. (Fig. 2).

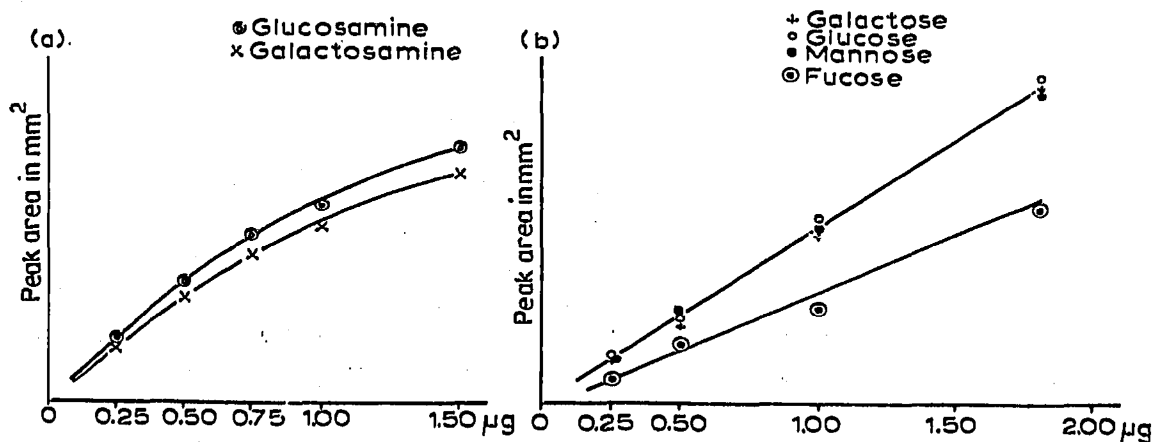

Fig. 2. (a) Standard curve for the estimation of hexosamines, obtained as described in the text. Abscisse: μg of hexosamine applied to the sheet. Ordinate: area of the densitometric recording in mm^2. (b) Standard curve for the estimation of hexoses.

The color given by fucose is less intense than the color given by the hexoses. The upper part of the chromatogram has a yellowish background after the development of the color, therefore, the reproducibility of the fucose determinations is less satisfactory. A colorless background can be obtained by using methanolic potassium hydroxide instead of methanolic sodium hydroxide for the alkalinization of the TTC reagent. However, in this case, fucose gives only about one fifth of the color intensity of the hexoses.

The results of an analysis of five samples of a 1:1:1:1 mixture of 1.5 g each of galactose, glucose, mannose and fucose are shown in Table I. The standard errors of the means were a few percent for the hexoses and about 6 % for fucose. The ratios of the different sugars were remarkably constant.

TABLE I

THE RATIO OF GALACTOSE, GLUCOSE, MANNOSE AND FUCOSE IN A TYPICAL RUN OF FIVE SAMPLES ON THE SAME SHEET

Sample No.	*Galactose*	*Glucose*	*Mannose*	*Fucose*
1	1.00	1.08	0.95	0.82
2	1.02	1.10	1.02	0.67
3	0.95	1.02	0.95	0.61
4	1.06	1.07	1.06	0.82
5	1.03	1.08	0.97	0.74
Amount added (μg)	1.5	1.5	1.5	1.5
Standard error of the mean	± 1.43 %	± 2.37 %	± 1.70 %	± 5.4 %

The formazan method is widely used for the detection and determination of reducing sugars on paper chromatograms[11]. The major inconvenience of this very sensitive method is a relatively strong and non-uniform background color. We have found that under the conditions described, it is possible to obtain an almost colorless background in the region of the hexoses on the Kodak 511 V sheets. For the densitometric recording, a methylene chloride vapor treatment makes the sheets more transparent, but equally good results were also obtained without this treatment.

For the quantitative determination of the hexoses in unknown samples, standard sugar mixtures were always run on the same sheet.

Amino acids and amino sugars, in the amounts that occur in the hydrolysate of the glycopeptides did not interfere with the analysis of neutral sugars.

The method was controlled by the determination of the ratio of the hexoses in the fibrinoglycopeptide mixture[13] and in ovomucoid[14].

Essentially the same value was found as given by the cited authors, the ratio of the mannose to galactose being 1.4:1 (result given by the authors[13]: 1.35:1) in the fibrinoglycopeptides, and 4.8:1 (result given by the authors[14]: 5:1) in ovomucoid.

We used this method for the estimation of the hexose ratio in glycopeptides isolated from the structural glycoproteins of connective tissue[7,8] (see Fig. 1).

The results obtained are for galactose–mannose 3:5 for a glycopeptide fraction

of the porcine aorta, and galactose–glucose–mannose 4:1:7 for the glycoprotein obtained from the rabbit cornea. (For more details see refs. 7 and 8.)

The method described is rapid and simple, and it may be generally used for the determination of neutral sugars in different hydrolysates and mixtures.

Determination of hexosamines. An identical procedure was used for hexosamine determination. Glucosamine and galactosamine are well resolved with the solvent described (see Fig. 1d). The color yield of glucosamine is somewhat stronger than that of galactosamine (ratio: 1:0.9 and 1:0.8–0.7) to that of galactose. The standard curve obtained with glucosamine and galactosamine in a typical experiment is shown in Fig. 2.

Though it is not linear, it can very well be used for quantitative estimation up to 1.5 μg. Amino acids in quantities usually found in glycopeptide preparations (about five times the amount of hexosamines) did not interfere with the separation and determination.

The standard error of repeated determinations was similar to that given for the hexoses ($\pm$ 2 %) of the mean of 5 determinations.

Application of this procedure for the estimation of hexosamines in the glycopeptides of structural glycoproteins have been described elsewhere in detail[7,8].

Laboratoire de Biochimie du Tissu Conjonctif,
5ter, rue d'Alésia, Paris 14e (France)

E. Moczar
M. Moczar
G. Schillinger
L. Robert

1 G. S. Marks, R. D. Marshall, A. Neuberger and G. Papkoff, *Biochim. Biophys. Acta*, 63 (1962) 340.
2 R. L. Whistler and J. N. BeMiller in R. L. Whistler and M. L. Wolfrom (Editors), *Methods in Carbohydrate Chemistry*, Vol. I, Academic Press, New York, 1962, p. 395.
3 R. G. Spiro, *J. Biol. Chem.*, 235 (1960) 2860.
4 M. L. Wolfrom, R. M. De Lederkremer and G. Schwab, *J. Chromatog.*, 22 (1966) 474.
5 J. M. Richey, H. G. Richey and J. Schraer, *Anal. Biochem.*, 9 (1964) 272.
6 C. H. Bolton, J. Clamp and L. Hough, *Biochem. J.*, 96 (1965) 5c.
7 E. Moczar and L. Robert, in preparation.
8 M. Moczar and L. Robert, in preparation.
9 L. Robert, J. Parlebas, P. Oudea, A. Zweibaum and B. Robert, in G. R. Tristram (Editor), *Biochemistry of Connective and Skeletal Tissues*, Butterworths, London, 1965, p. 406.
10 A. Neuberger and R. D. Marshall, in A. Gottschalk (Editor) *Glycoproteins*, Elsevier, Amsterdam, 1966, p. 190.
11 R. Tinelli, *Bull. Soc. Biol.*, 48 (1966) 182.
12 F. G. Fischer and H. Doerfel, *Z. Physiol. Chem.*, 297 (1954) 164.
13 L. Mester, E. Moczar, G. Wass and L. Szabados, *Compt. Rend.*, 260 (1965) 2342.
14 J. Montreuil, A. Adam-Chausson and G. Spik, *Bull. Soc. Chim. Biol.*, 47 (1965) 1867.

Received June 7th, 1967

Note added in proof

We learned recently that a similar method for hexose and hexosamine determination by paper chromatography is being used in Dr. Mester's laboratory (L. Mester, G. Vass, L. Szabados and A. Schimpl, *FEBS 1967 Meeting, Oslo*, Abstr. 575, p. 141).