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AUTOMATIC AMINO ACID AND SUGAR ANALYSIS OF GLYCOPROTEINS

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SUMMARY

Amino acid analyzers were used for complete analyses of glycoproteins. The methods of analysis used were cation-exchange chromatography for amino acids, phosphoamino acids and amino sugars with ninhydrin as reagent, and anion-exchange chromatography for neutral sugars with copper Bicinchoninate as reagent. A separate program was developed for the analysis of charged sugar components, N-acetylneuraminic acid and amino sugars in one chromatogram. The possibility of detection of radioactively labelled components is also demonstrated. The detection limits and possibilities for column and reactor switches are discussed.

INTRODUCTION

The emergence of the amino acid analyzer provided the initial basis for the automatic analysis of amino acids. Subsequently, with the development of appropriate supports and reagents, the range of this instrument was extended to include the automation of the analysis of neutral and charged sugar components[1]. The present paper describes a program for the sequential analysis of the amino acid, sugar, and phosphoamino acid content of glycoproteins.

MATERIALS AND METHODS

Hydrolysis

The analysis strategy and hydrolysis conditions are shown in Fig. 1.

Amino acid and phosphoamino acid analyses were performed with a LC-5000 automatic amino acid analyzer (Biotronik, Frankfurt/Main, F.R.G.). For the analyses of amino acids and amino sugars, programs with sodium or lithium buffer were used (in accord with the Biotronik manual). Conditions for analysis of phosphoamino acids: 0.08 *M* lithium citrate buffer, pH 1.7 (adjusted with HCl); column temperature 45°C, without pre-column. The sample was dissolved in a 0.05 *M* lithium citrate buffer, pH 1.5 (adjusted with HCl).

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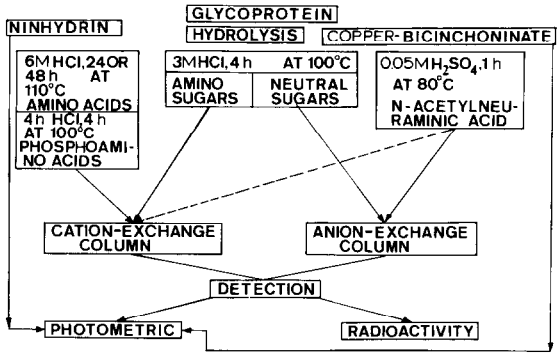


Fig. 1. Scheme of glycoprotein analysis.

In all cases, ninhydrin was used as reagent, the reaction temperature was 121°C, and detection was photometric at 570 and 440 nm. Other conditions: separation column, 250 × 3.2 mm, BT2710 cationic exchanger (Biotronik), bed height 140 mm; prewash column, 50 × 6 mm, BTC-F (Biotronik), bed height 28 mm; pump flow, 19 ml/h for buffer, 10 ml/h for reagent.

For the amino sugar and N-acetylneuraminic acid analyses in one chromatogram the same column was used as for amino acids. Other conditions: buffer, 0.1 *M* sodium citrate, pH 7.2; reagent, copper bicinchoninate; temperature, 40°C for first 15 min then 63°C; flow-rate of both buffer and reagent, 10 ml/h; detection, photometric at 570 nm.

The neutral sugars were determined with a LC-6001 amino acid analyzer (Biotronik), equipped with an anion-exchanger column. Borate buffers were used: A, 0.4 *M* potassium borate, pH 7.6; B, 0.7 *M* potassium borate, pH 9.2. For column regeneration 10% potassium tetraborate (w/v) was used. Other conditions: separation column, 250 × 6 mm, DA-X8-11 anionic exchanger (Biotronik), bed height 170 mm; prewash column, 95 × 9 mm, AG-X1-4 (Biotronik); bed height 170 mm; column temperature 63°C; pump flow, 40 ml/h for both buffer and reagent; reagent, copper bicinchoninate; reaction temperature, 100°C; detection at 570 nm.

Radioactively labelled sugars were D-[1-^{3}H]galactose (10 mCi/mmol) and D-[3-^{3}H]-glucose (5 mCi/mmol) (Amersham, Arlington Heights, IL, U.S.A.). For one analysis, 1000 cpm of each sugar were taken. Detection: the fractions (0.5 ml each) were collected (SuperRac fraction collector; LKB, München, F.R.G.) and the radioactivity was determined (LB-230 liquid scintillator; Beckman, San Diego, CA, U.S.A.).

Chemicals

All chemicals were analytical grade and were obtained either from Merck (Darmstadt, F.R.G.) or Serva (Heidelberg, F.R.G.).

RESULTS AND DISCUSSION

The complete analytical scheme is shown in Fig. 1. Although a combined amino acid and sugar analysis may be performed on a single instrument by making

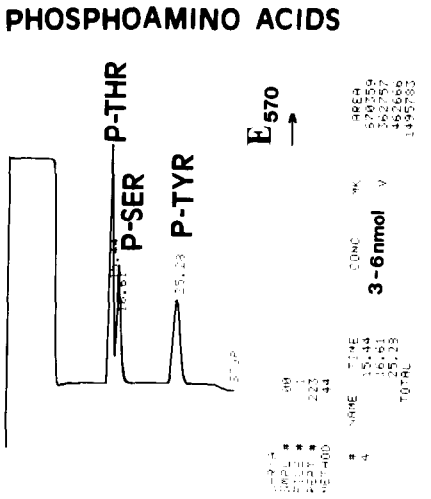


Fig. 2. Separation of phosphoamino acids. For conditions see Materials and methods section.

the appropriate column changes, it is more convenient, as here, to employ two analyzers alternatively provided with anionic and cationic exchange columns.

For the resolution of amino acids, the previously described sodium and lithium buffer systems[2] proved satisfactory. However, the strongly acidic hydrolytic conditions employed caused partial destruction of amino sugars. In the sodium system, the non-degraded amino sugars appear as peaks after phenylalanine; in the lithium system, they are intermediate between alanine and valine.

Using ninhydrin as indicator, the detection limit is about 100 pmol and the optimal sample concentration is 1.0–5.0 nmol for each amino acid. The phosphoamino acid analyses of Cohen-Solal *et al.*[3] and Vandlen *et al.*[2] were modified to permit the identification of phosphotyrosine. The resolution achieved is shown in Fig. 2.

In a single chromatogram on a cationic exchanger, N-acetylneuraminic acid

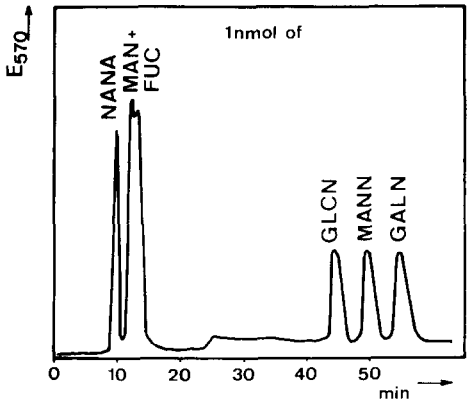
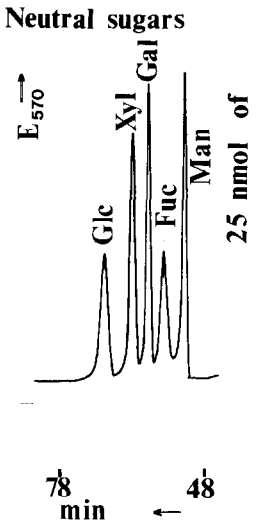


Fig. 3. Separation of N-acetylneuraminic acid and amino sugars with a cation-exchange column. N-Acetylneuraminic acid is excluded by the column and appears before neutral sugars; the amino sugars are well separated.



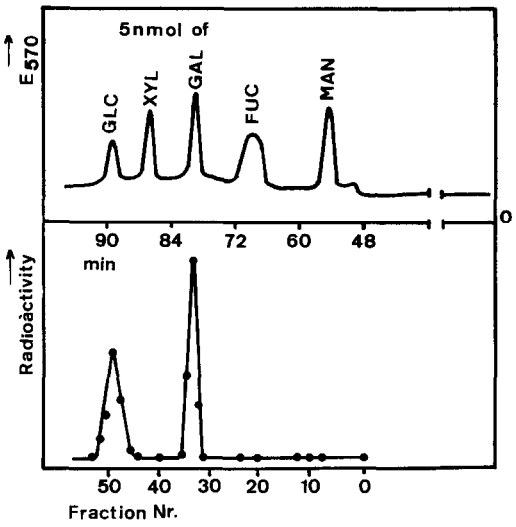


Fig. 4. Separation of the neutral sugars, mannose, fucose, galactose, xylose and glucose.

Fig. 5. Detection of the radioactively labelled sugars glucose and galactose. The fractions were collected and their radioactivity was determined. The results are shown in the lower part of the figure.

was excluded from the column and appeared before the neutral sugars and the amino sugars, glucosamine, galactosamine and mannosamine, all of which were well separated (Fig. 3). With copper bicinchoninate as the monitoring reagent, the detection limit is 200 pmol; optimum amounts range from 2.5 to 10 nmol. However, care must be taken when the analysis of amino acids and sugars is carried out with a single column. Under these conditions, the detergent Brij in the buffer system must be washed out in order to prevent the formation of a precipitate with copper bicinchoninate and a resultant obstruction of the reactor. If possible, this problem is best avoided by the use of separate analyzers.

The resolution of different types of neuraminic (sialic) acids can be achieved on a short anion-exchange column[4,5].

The results achieved with several individual monosaccharides are shown in Fig. 4. For mannose, galactose and xylose, the detection limit is about 1 nmol; for glucose and fucose, about 2 nmol. The optimum amount of each sugar is between 5 and 25 nmol. Greater sensitivity is achieved by the use of radioactive sugars, as

TABLE I

SUGAR ANALYSIS OF TRANSFERRIN (Tf)

Sugar	*nmol per 5 mg Tf*	*Ratio*
NeuNAc	227	1.1
Gal	198	1.0
GlcNAc	407	2.1
Man	337	1.5

TABLE II

AMINO ACID ANALYSES OF THE OLIGOSACCHARIDE-CONTAINING FRACTION AFTER HYDRAZINOLYSIS OF TRANSFERRIN

Amino acid	*nmol per 5 mg Tf*	
	Before hydrolysis	*After hydrolysis*
Asp	10	128
Ala	7	114
Ser	23	32
Glu	–	32
Thr	14	26
Gly	17	25

shown in Fig. 5 for glucose and galactose. Flow detectors are now available for direct monitoring of radioactivity.

Application to control of hydrazinolysis of transferrin

A 5-mg amount of transferrin was subjected to hydrazinolysis, hydrazine was removed under reduced pressure and the product was re-acetylated with acetic anhydride. The oligosaccharides were partially purified by chromatography on a Bio-Gel P-2 and Concanavalin A column, followed by paper chromatography[6].The oligosaccharides were eluted with water and hydrolyzed with either 0.025 *M* sulphuric acid (80°C, 1 h) or 3 *M* hydrochloric acid (100°C, 3 h, *cf.*, also Fig. 1). The results of the sugar analyses are shown in Table I.

In order to evaluate the completeness of hydrazinolysis the oligosaccharide-containing fractions were either directly applied to the amino acid analyzer or were subjected to acid hydrolysis. The results, shown in Table II, clearly demonstrate that by far the major part (about 90%) of the oligosaccharides was still bound to asparagine.

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