Journal of Chromatography, 167 (1978) 197–203

CHROM. 11,162

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC INVESTIGATION OF THE AMINO ACID, AMINO SUGAR AND NEUTRAL SUGAR CONTENT IN GLYCOPROTEINS

MIKHAEL M. TIKHOMIROV and ANATOLIY Ya. KHORLIN

U.S.S.R. Academy of Sciences, Shemyakin Institute of Bioorganic Chemistry, Vavilova 32, 117312 Moscow (U.S.S.R.)

and

WOLFGANG VOELTER and HERMANN BAUER*

Institut für Organische Chemie der Universität Tübingen, Auf der Morgenstelle 18, 7400 Tübingen (G.F.R.)

SUMMARY

A method for the simultaneous separation and determination of amino acids, amino sugars and neutral carbohydrates is described. Stepwise elution systems with sodium citrate and borate buffers have been developed for the ion-exchange liquid chromatographic separation of amino acids and sugars, using 8-μm particle size resins and the Stein and Moore and orcinol colorimetric method for detection.

With the aid of this system, the direct quantitative comparison of sugars and amino acids by liquid chromatography becomes possible for the first time.

INTRODUCTION

In recent years there has been increasing interest in the study of glycoproteins, because of their fundamental biological importance. Particular attention has been focused on the elucidation of the structure of the carbohydrate units and on their attachment to the peptide chains. Various hydrolytic, chromatographic and colorimetric techniques for the release and identification of the amino acids and sugars of glycoproteins have been described[1].

At present, there are several liquid chromatographic methods available, utilizing ion-exchange resins for the quantitative analysis of amino acids[2] and sugars[3]. The main drawback of these chromatographic procedures is the necessity for two different separations, one for the determination of amino acids and another for sugars.

This paper deals with a method for the automated quantitative analysis of amino acids and sugars using ion-exchange resins for the separation of these two classes of substances first. The further separation into components is performed on two different columns. Neutral sugars and basic compounds are separated immedi-

* To whom correspondence should be addressed.

ately after injection with the aid of a small pre-column, which is filled with a strongly acidic, protonated ion-exchange resin. Basic components are retarded, while neutral sugars pass without interaction and thus can be transferred to the carbohydrate column. Amino acids are then eluted with sodium citrate buffers and brought on to the amino acid column. Separation of the sugars and amino acids and the successive analysis of the different compounds is controlled with a high-pressure stable motor valve.

EXPERIMENTAL

Apparatus

These investigations were performed with a Biotronik liquid chromatograph. The chromatographic system is shown schematically in Fig. 1.

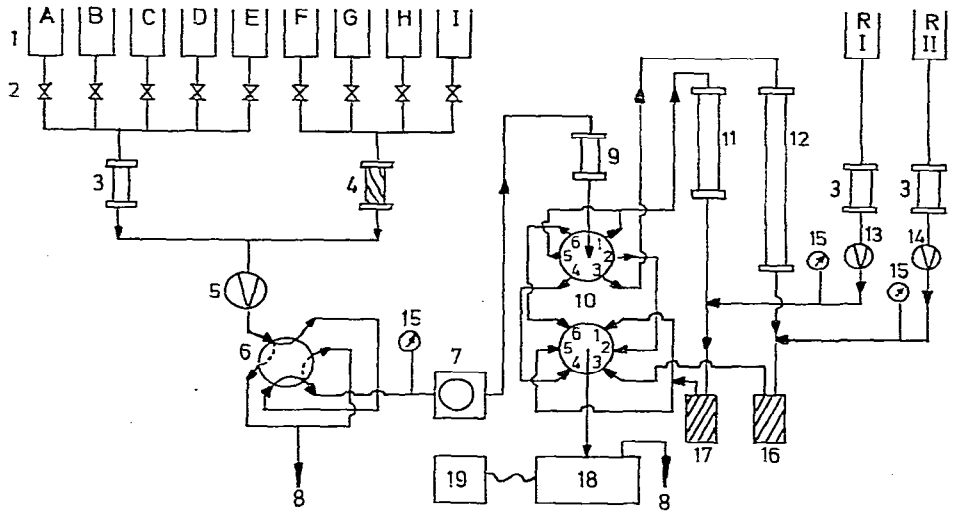

Fig. 1. Schematic flow scheme of the combined amino acid–sugar analyser. (1) Eluent reservoirs; (2) electric valves; (3) bubble trap; (4) ammonia filter; (5) Milton-Roy Dosapro mini-pump; (6) manual valve; (7) sample injection; (8) out-flow; (9) pre-column; (10) motor valve; (11) DA-X-8-8 column for sugars; (12) DC-8-9 column for amino acids and amino sugars; (13) modified acid-resistant Milton-Roy mini-pump (orcinol–H_2SO_4); (14) ninhydrin pump; (15) pressure gauge; (16) 30 m × 0.3 mm PTFE reaction coil (100°); (17) 20 m × 0.5 mm PTFE reaction coil (100°); (18) two-channel photometer (420 and 570 nm); (19) recorder.

The 0.3 × 2 mm transport lines on the pressure side were made from PTFE tubes and are resistant up to 120 bar.

Amino acids and sugars were separated in a small pre-column (9) of length 5 mm and I.D. 4 mm. Chromatographic analyses were performed with a 250 × 4 mm column for amino acids (12) and a 160 × 4 mm column for sugars (11), both being high-pressure stable tensile discharged glass columns (maximum pressure 120 bar). The columns were glass jacketed and connected with a constant-temperature water circulator.

A high-pressure stable motor valve (10) from a commercially available automated sampler was used after modification. For the development of the colour reactions adjustable heating baths, equipped with 30 m × 0.3 mm (for amino acids) and 20 m × 0.5 mm (for sugars) PTFE coils were installed. Detection was carried out with a two-channel filter photometer (18) at 420 and 570 nm and a cell of path length 16 mm.

All time-dependent processes of separation were controlled by a microprocessor with 15 time stores and 20 functions per store.

Resins

For ammonia a Dowex 50W-X8 resin (50–100 mesh) filter was used. The pre-column and the amino-acid column were filled with DC-8-9 resin (Durrum, Palo Alto, Calif., U.S.A.), which is an 8% cross-linked sulphonated polystyrene of particle size 8 ± 2 μm. The sugars were separated on DA-X-8-8 resin (Durrum), which is an 8% cross-linked basic polystyrene of particle size 8 ± 2 μm.

Chemicals and reagents

Sugars and amino acids of the highest available purity were obtained from Merck (Darmstadt, G.F.R.). Boric acid, sulphuric acid, orcinol, sodium citrate, sodium chloride, hydrochloric acid, cellusolve and ninhydrin were of analytical-reagent grade (Merck).

The buffers were prepared by dissolving boric acid, sodium citrate and sodium chloride in doubly distilled and deionized water and adjusting the pH to the desired value with concentrated sodium hydroxide solution or hydrochloric acid.

For the regeneration of the sugar column 10% potassium tetraborate solution, for the amino acid column 0.5 *N* sodium hydroxide solution and for the pre-column 0.5 *N* hydrochloric acid were used.

As reagents for the colour-reactions orcinol (0.1%) in concentrated sulphuric acid and the Stein and Moore reagent were applied.

Procedure

All important data for the separation procedure are given in Table I. After the injection of the sample on the pre-column, neutral sugars were eluted with water and transferred to the DA-X-8-8 column in borate form. After 5 min all neutral sugars are eluted and then all connections from the buffer valves to the pre-column were rinsed with starting buffer for the amino acid separation. A period of 10 min proved to be sufficient for rinsing and converting the pre-column into the sodium form without releasing the amino acids. This procedure was necessary in order to avoid disturbing the equilibrium conditions of the DC-8-9 column. Thus the amino acids and amino sugars could be separated after changing the flow to the DC-8-9 column with high resolution and sensitivity under normal separating conditions. Before changing the flow to the DA-X-8-8 column, a further rinsing step with starting buffer for neutral sugars was applied.

After the separation of the sugars the pre-column was converted to the H^+ form with 0.5 *N* hydrochloric acid and equilibrated with water.

TABLE I

DATA FOR SEPARATION PROCEDURE

Step	*Flow direction*	*Eluent*	*Position of motor valve*	*Temperature of columns (°C)*	*Reagent*	*Time (min)*
1	To DA-X-8-8 column	Water (E)	1	45	—	5
2	To waste	0.2 *N* sodium citrate buffer, pH 3.25 (F)	2	45	—	4
3	To waste	0.2 *N* sodium citrate buffer, pH 3.25 (F)	2	45	—	6
4	To DC-8-9 column	0.2 *N* sodium citrate buffer, pH 3.25 (F)	3	45	Ninhydrin	30
5	To DC-8-9 column	0.2 *N* sodium citrate buffer, pH 4.25 (G)	3	59	Ninhydrin	45
6	To DC-8-9 column	1.6 *N* sodium citrate buffer, pH 4.5 (H)	3	68	Ninhydrin	45
7	To DC-8-9 column	0.5 *N* sodium hydroxide solution (I)	3	62	—	30
8	To DC-8-9 column	0.2 *N* sodium citrate buffer, pH 3.25 (F)	3	45	—	30
9	To waste	0.2 *M* borate buffer, pH 8.3 (A)	4	45	—	5
10	To waste	0.2 *M* borate buffer, pH 8.3 (A)	4	45	—	10
11	To DA-X-8-8 column	0.2 *M* borate buffer, pH 8.3 (A)	5	45	Orcinol–H_2SO_4	60
12	To DA-X-8-8 column	0.4 *M* borate buffer, pH 9.4 (B)	5	68	Orcinol–H_2SO_4	135
13	To DA-X-8-8 column	0.2 *M* borate buffer, pH 8.3 (A)	5	45	—	30
14	To waste	0.5 *N* hydrochloric acid (D)	6	45	—	15
15	To waste	Water (E)	6	45	—	30

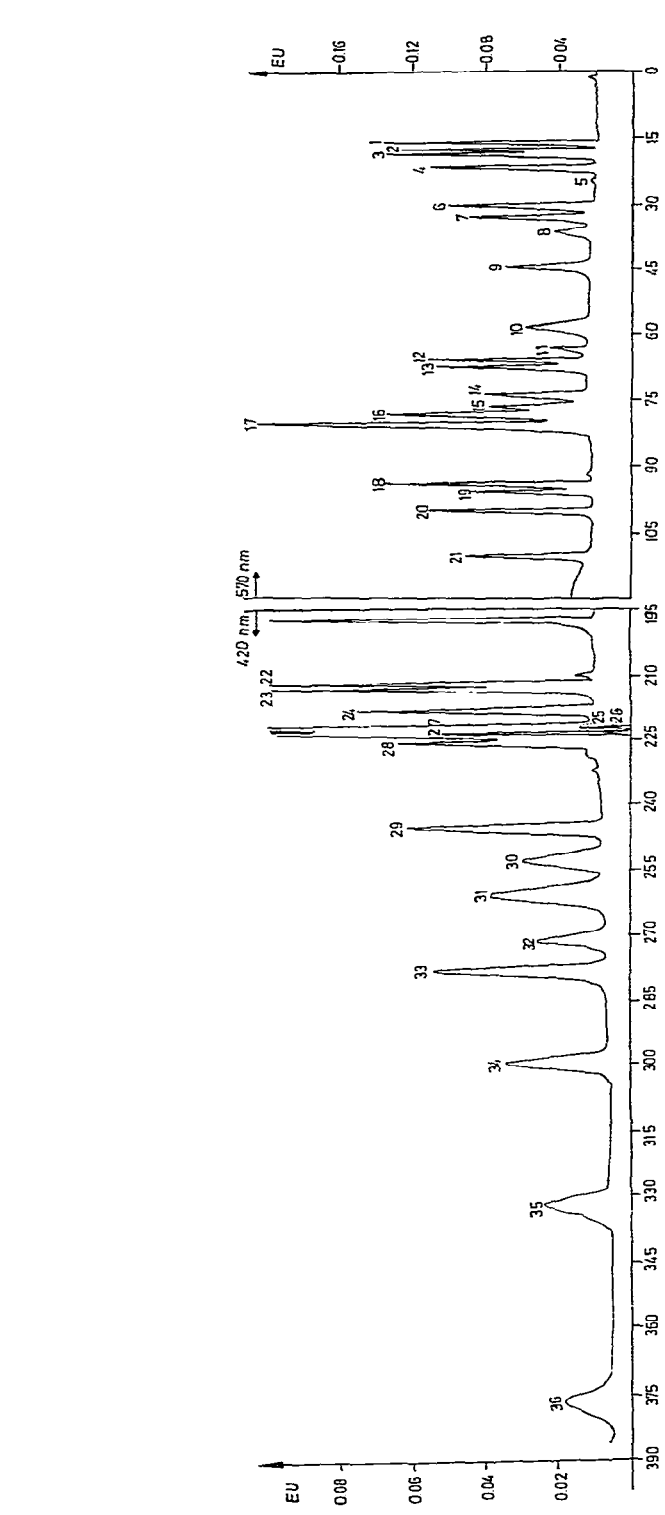

Fig. 2. Chromatogram of a mixture of amino acids, amino sugars and neutral sugars. Pre-separation column, 5 × 4 mm, DC-8-9 resin; amino acid and amino sugar column, 250 × 4 mm, DC-8-9 resin; neutral sugar column, 160 × 4 mm, DA-X-8-8 resin; flow-rate, 0.24 ml/min; pressure, 80–110 bar; recorder range, 0–0.2 A (ninhydrin, 570 nm) or 0–0.1 A (orcinol–sulphuric acid, 420 nm); for further separation conditions, see Table I. Peaks: 1 = aspartic acid; 2 = threonine; 3 = serine; 4 = glutamic acid; 5 = proline; 6 = glycine; 7 = alanine; 8 = cystine; 9 = valine; 10 = methionine; 11 = unknown; 12 = isoleucine; 13 = leucine; 14 = tyrosine; 15 = phenylalanine; 16 = galactosamine; 17 = glucosamine; 18 = lysine; 19 = ammonia; 20 = histidine; 21 = arginine; 22 = sucrose; 23 = trehalose; 24 = cellobiose; 25 = maltose; 26 = rhamnose; 27 = lactose; 28 = ribose; 29 = mannose; 30 = fructose; 31 = arabinose; 32 = galactose; 33 = xylose; 34 = glucose; 35 = gentiobiose; 36 = melibiose; 37 = fucose. Each peak represents 2.05 nmole per amino acid and 4.40 nmole per amino sugar and neutral sugar.

RESULTS AND DISCUSSION

Fig. 2 shows a chromatogram of a mixture of amino acids, amino sugars and neutral sugars, which was obtained with the combined amino acid–carbohydrate analyser. Each peak represents 2.05 nmole per amino acid and 4.40 nmole per sugar.

Comparison of the sensitivity and resolution of this combined system with separate analytical procedures for sugars and amino acids demonstrates clearly the advantage of the simultaneous determination of carbohydrates and amino acids. On the one hand only half the amount of sample is necessary for the analysis, which is of importance if only small amounts of isolated substances are available, and on the other hand the direct quantitative comparison of sugars with amino acids becomes possible without an additional internal standard.

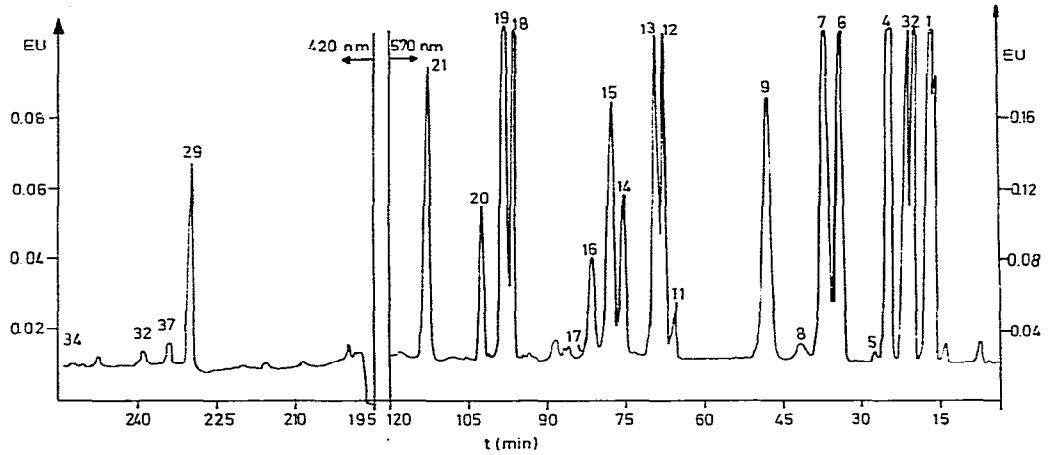

Fig. 3. Chromatogram of a hydrolysate of ovalbumin. The sugar column was reduced in size to 65 × 4 mm. Neutral sugars were eluted isocratically (0.5 *M* boric acid, pH 8.5). For further separation conditions see Fig. 2 and Table I. Amount injected, 7.8 μg.

Fig. 3 shows a chromatogram of 7.8 μg of a hydrolysate of ovalbumin that was optimized especially for the elucidation of the sugar content in glycoproteins. The DA-X-8-8-column was reduced in size to 65 × 4 mm. For isocratic elution a 0.5 *M* boric acid buffer of pH 8.5 was used. The hydrolysis was performed in 6 *N* trifluoroacetic acid at 100° for 36 hours. Contrary to the usual conditions for hydrolysis in 6 *N* hydrochloric acid, which destroys all sugars completely, the degradation of the amino sugars and neutral sugars was less than 20%. The yield of amino acids compared with the hydrochloric acid method is nearly the same (± 5%) except for cystine and methionine, which are nearly fully recovered.

ACKNOWLEDGEMENTS

We thank Biotronik GmbH, Frankfurt, G.F.R., for supporting this work. Mikhael M. Tikhomirov thanks the Deutsche Forschungsgemeinschaft for a fellowship in Tübingen.

REFERENCES

1 A. Gottschalk (Editor), *Glycoproteins, Parts A and B*, Elsevier, Amsterdam, 2nd ed., 1972.
2 D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, 30 (1958) 1190.
3 H. Bauer and W. Voelter, *Chromatographia*, 9 (1976) 433.