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Note

Rapid chromatographic analysis of neutral oses and hexosamines of the human erythrocyte membrane

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The carbohydrates of glycoproteins and glycolipids from human erythrocyte membrane can be divided into three groups, neutral oses, hexosamines and sialic acids, and there are numerous methods for their determination after hydrolysis. For neutral oses, the most widely used methods are gas–liquid chromatography (of trimethylsilyl ethers[1,2] or alditol acetates[3–5]) and ion-exchange chromatography. In the latter instance, two basic techniques have been devised: differential partitioning of the sugars between anion- or cation-exchange resins and ethanol–water eluent according to Samuelson and co-workers[6–8], and chromatography of sugar–borate complexes on basic ion-exchange resins in the borate form according to Zill and co-workers[9,10]. For hexosamines, the most widely used technique is amino acid chromatography[11,12].

This paper reports the development of rapid, reproducible and sensitive separations of neutral oses and hexosamines hydrolysed from human erythrocyte membranes.

EXPERIMENTAL

Apparatus

The work was performed with an N.C.I. Technicon AutoAnalyzer, which consists of the following Technicon modules: (a) Dosapro positive micro-pump; (b) jacketed chromatographic column; (c) peristaltic pump; (d) heating bath; (e) colorimeter with 15-mm light-path cuvette; and (f) three-channel recorder. For neutral oses, the chromatographic column (140 × 0.3 cm), was thermostated at 77° with water from a Colora K_5 ultra-thermostat (Messtechnik, Lorch/Würt., G.F.R.). For hexosamines, the chromatographic column (130 × 0.6 cm) was thermostated at 55° with water from a Haake Type FE thermostat (Berlin Lichterfelde, G.F.R.). The chromatographic systems and analytical manifolds for neutral oses and hexosamines are shown schematically in Fig. 1.

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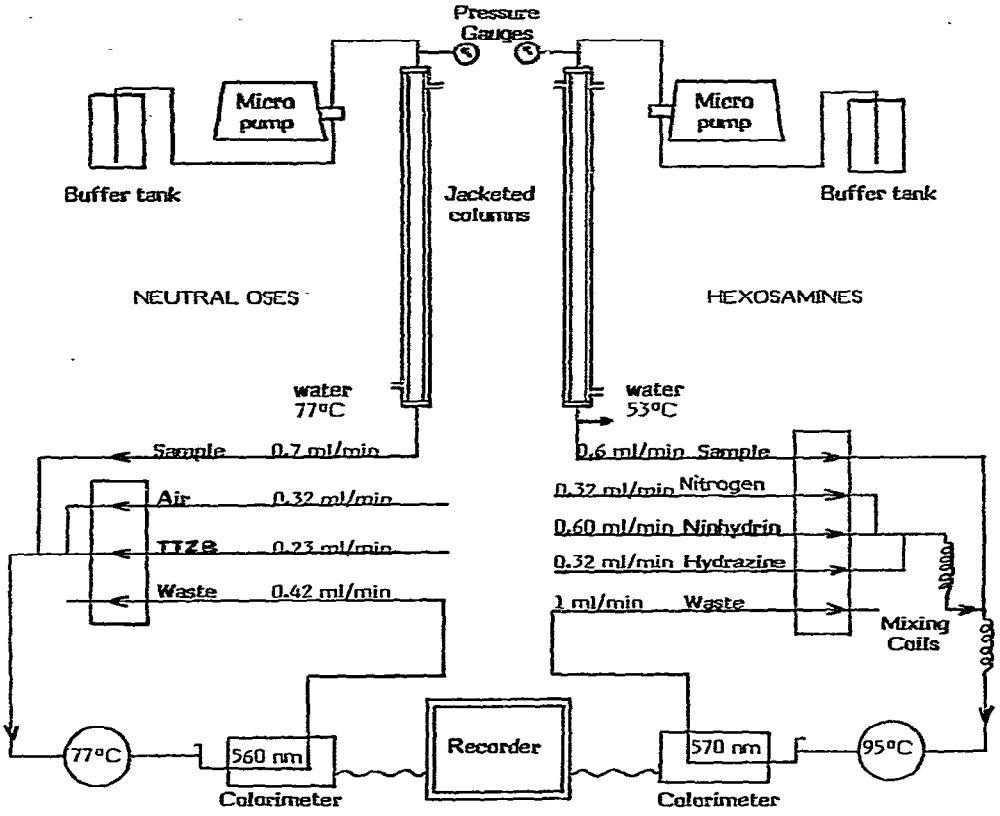

Fig. 1. Schematic diagram of the chromatographic and analytical systems. TTZB = tetrazolium blue.

Resins

The resins, supplied by Technicon, were Type S Chromobeads anion-exchange resin for neutral oses and Type A Chromobeads cation-exchange resin for hexosamines.

Reagents and materials

L-Fucose and L-norleucine (A grade) were purchased from Calbiochem (Los Angeles, Calif., U.S.A.). D-Mannose, D-galactose, D-glucose, L-rhamnose and D-glucosamine hydrochloride (for biochemistry) and ninhydrin, hydrazinium sulphate and sodium sulphate were obtained from E. Merck (Darmstadt, G.F.R.). D-Galactosamine hydrochloride (grade I) and tetrazolium blue were supplied by Sigma (St. Louis, Mo., U.S.A.).

For neutral oses, a standard was prepared by dissolving 0.1 g each of fucose, mannose, galactose and glucose in 100 ml of water; a 0.1% solution of rhamnose in water was used as the internal standard. The eluent was prepared by diluting absolute ethanol to 84% (w/w) with distilled water. The dye consisted of 0.5 g of tetrazolium blue in 1 l of a solution consisting of 10 *N* sodium hydroxide solution–84% ethanol–water (20:480:500).

For hexosamines, a standard was prepared by dissolving 0.1 g of glucosamine hydrochloride, 0.1 g of galactosamine hydrochloride and 0.05 g of norleucine in 100 ml of water; a 0.05% solution of norleucine was used as the internal standard. The eluent was a 0.06 *M* sodium citrate buffer containing 0.34 *M* sodium chloride and 5% methanol (pH 5.7).

Methods

Neutral oses. Aliquots of standard (usually 50 μl of a 1:10 dilution in 84% ethanol) or hydrolysed samples (250 μl or more), *i.e.*, about 2 μg of each sugar, were injected with a microsyringe. The column was filled with 84% ethanol. The eluent pump was set at 0.7 ml/min and the pressure was 500–600 lb/in.^{2}. A 50-μl volume of rhamnose standard, diluted 1:10 with 84% ethanol, was injected as an internal standard 10 min before the samples. After two chromatographic runs, the resin was washed, at the same flow-rate, with water, then with 0.5 *M* sodium sulphate solution and with water again, for 30 min each time, and conditioned with 84% ethanol.

Hexosamines. Aliquots of standard (usually 100 μl of a 1:10 dilution in citrate buffer) or hydrolysed samples (500 μl), *i.e.*, about 25–50 μg of each hexosamine, were injected with a microsyringe. The column was filled with the 5% methanol–citrate buffer. The eluent pump was set at 0.75 ml/min and the pressure was 300–400 lb/in.^{2}. A 100-μl volume of norleucine standard, diluted 1:10 with the same buffer, was injected as an internal standard 20 min after the samples. After each chromatographic run, the resin was washed with 0.2 *N* sodium hydroxide solution and conditioned with the citrate buffer.

RESULTS

Figs. 2 and 3 show the chromatograms of standard and hydrolysate, respectively, for neutral oses and hexosamines and also the elution time for each sugar.

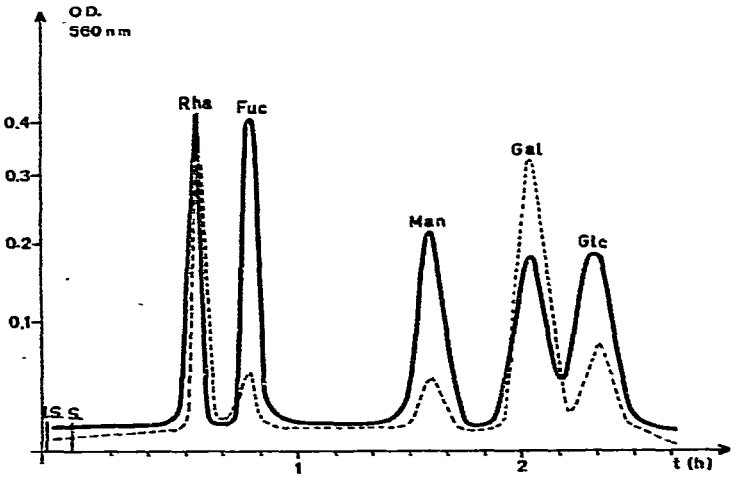

Fig. 2. Typical chromatograms of neutral oses: standard (continuous line) and hydrolysate (broken line). Each peak of the standard represents 5 μg of the following sugars: rhamnose (Rha), fucose (Fuc), mannose (Man), galactose (Gal) and glucose (Glc). IS = injection of rhamnose (5 μg) as internal standard; S = injection of standard or sample.

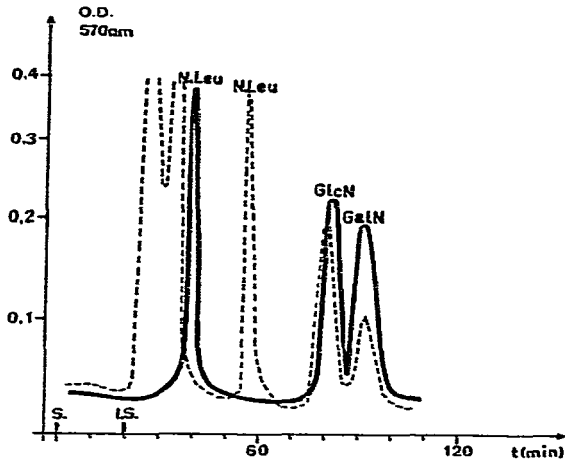

Fig. 3. Typical chromatograms of hexosamines: standard (continuous line) and hydrolysate (broken line). Each peak of the standard represents 5 μg of norleucine (N.Leu) and 10 μg each of glucosamine (GclN) and galactosamine (GalN). IS = injection of N.Leu solution (5 μg) as internal standard; S = njection of standard or samples.

In the quantitative evaluation of the chromatograms, the areas of the chromatographic peaks (absorbance *versus* time) were usually integrated by multiplying the height (net absorbance) of the peak by the width (millimetres) at half of the net height[13]. A sugar to internal standard colour ratio was determined for each carbohydrate. The reproducibility of the two methods, evaluated from these ratios, is indicated in Table I. The sensitivity, without special amplification, is about 0.7–1 μg, *i.e.*, 4–6 n*M*, for neutral oses and about ten times lower for hexosamines.

TABLE I

REPRODUCTIBILITY OF SUGAR TO INTERNAL STANDARD COLOUR RATIOS

The internal standards are rhamnose for neutral oses and norleucine for hexosamines.

Sugar	*n*	*M* ± *S.D.*	*Coefficient of variation (%)*
Fucose	24	1.236 ± 0.049	4.0
Mannose	23	1.155 ± 0.077	6.7
Galactose	23	1.284 ± 0.08	6.2
Glucose	23	1.435 ± 0.092	6.4
Glucosamine	11	0.585 ± 0.04	7.0
Galactosamine	11	0.660 ± 0.03	5.0

Table II gives the mean total sugar composition of human erythrocyte membrane obtained by means of these techniques. It is of the same order of magnitude as the compositions described by Winzler[14,15], who used gas chromatographic methods, and Relander[16], who determined only galactose and glucose with their specific oxidases and total hexosamines by Elson–Morgan assay.

TABLE II

CARBOHYDRATE CONTENT OF HUMAN ERYTHROCYTE MEMBRANE

Results are expressed as micrograms per milligram of dried membrane. The mean values were calculated from the 34 erythrocyte samples independently of their A, B or O blood group.

Sugar	$M \pm S.E.M.$	*n*
Fucose	1.3 ± 0.050	34
Mannose	1.9 ± 0.038	34
Galactose	12.1 ± 0.344	34
Glucose	3.8 ± 0.320	34
Glucosamine	7.7 ± 0.360	21
Galactosamine	4.8 ± 0.280	21

DISCUSSION

The principal objective of the methods reported here was to obtain the best separations of the four neutral oses fucose, mannose, galactose and glucose and of the two hexosamines glucosamine and galactosamine that can be found in human erythrocyte membrane. Partition chromatography of neutral sugars between anion- or cation-exchange resins and ethanol–water eluents has been devised by Samuelson and co-workers[6–8] and various modifications have been described[17–19]. This chromatographic system presents some technical difficulties, as indicated by Floridi[20]: high temperature with volatile solvents, high pressure and rapid wearing out of the manifold tubes owing to the high concentration of ethanol in the eluate. As neither xylose nor arabinose exists in human erythrocyte membrane, there remain only four neutral oses to resolve. Therefore, the chromatographic procedure for neutral oses was modified. The use of 84% ethanol reduces the problem of volatility, and also results in an appreciable shortening of the chromatographic run to 160 min compared with 3–4 h as in the method described by Mopper and Degens[19]. Xylose and arabinose would be eluted between fucose and mannose and could easily be separated with more concentrated ethanol as eluent. The difficulty in relation to manifold tubes was resolved by (1) systematically changing the waste tube after every four chromatographic runs and (2) direct injection of the column eluate into the analytical flow, downstream of the peristaltic pump.

The method reported here gives good separations of hexosamines from each other and from amino acids within 90–100 min. The hexosamines are eluted after the aromatic amino acids and glucosamine before galactosamine. More rapid chromatographic resolutions can be achieved, but more sophisticated apparatus is required[12,21,22].

These methods have been developed for use in the systematic study of human erythrocyte membrane carbohydrate contents, in order to establish their possible modifications with blood groups, ageing or pathology. They can easily be used for the determination of the carbohydrate contents of all glycoproteins and glycolipids.

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