The Use of Gas-Liquid Chromatography in the Analysis of Neutral Monosaccharides in Hydrolysates of Gastric Mucopolysaccharides

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1. Conditions are described for the separation and estimation of the neutral monosaccharides obtained on acidic hydrolysis of human gastric mucopolysaccharides. 2. The technique involves the formation of the trimethylsilyl derivatives of the sugars and the analysis of these by gas-liquid chromatography. 3. The monosaccharides estimated in gastric mucopolysaccharides by this technique were L-fucose, D-mannose, D-galactose and D-glucose. 4. The analytical values for glucose and fucose obtained by this method agreed well with values obtained by the glucose oxidase and thioglycolic acid methods respectively. 5. Evidence is presented which clearly indicates that gas-liquid chromatography is a faster, more sensitive and more convenient technique for the measurement of these compounds than any other in use at present.

The identification and particularly the estimation of the monosaccharides produced during an acidic hydrolysis of polysaccharides derived from both animal and plant species has in the past been laborious and time-consuming. Typical techniques have involved the use of column- and paper-chromatographic procedures and the use of generally non-specific colour reactions with various phenols in concentrated sulphuric acid. These colour reactions do not usually distinguish between various monosaccharides and therefore have limited applicability for biological material, and furthermore lack high sensitivity.

The application of gas-liquid chromatography to carbohydrates has been hampered by the lack of suitable derivatives that are sufficiently volatile to chromatograph. The acetyl and methyl ethers of the sugars have been subjected to gas-liquid chromatography with some success, and a comprehensive survey of these separations has appeared (Bishop, 1962). More recently, work has been proceeding with the trimethylsilyl derivatives of carbohydrates and, although the results obtained were not particularly encouraging at first (Hedgeley & Overend, 1960; Witsch, 1961), Sweeley, Bentley, Makita & Wells (1963) have described the successful chromatographic separation of a large number of carbohydrate derivatives. Only the trimethylsilyl derivatives have been considered in the present work. The preparation of these is based on the method described by Sweeley *et al.* (1963), in which a very high yield of the trimethylsilyl derivative of α -methyl glucoside was obtained.

Subsequent workers (Wells, Chin & Weber,

1964; Perry, 1964) have obtained quantitative yields for the trimethylsilylation reaction with sugars and *N*-acetyl-amino sugars by using almost identical amounts of reagents and similar techniques.

EXPERIMENTAL

Materials

Pyridine (AnalaR grade; British Drug Houses Ltd., Poole, Dorset) was dried overnight with BaO and distilled. The distillate was kept over CaH₂ in an amber-coloured bottle.

Trimethylchlorosilane (reagent grade; Hopkin and Williams Ltd., Chadwell Heath, Essex) was used directly. Hexamethyldisilazane (L. Light and Co. Ltd., Colnbrook, Bucks.) was also used directly.

Dowex 2 (200–400 mesh) was treated as follows before use. The resin was suspended in *N*-NaOH, poured into a column and washed exhaustively with 2*M*-Na₂CO₃ until chloride-free. Washing was then continued with 5% (w/v) NH₄HCO₃ and finally the material was washed with water until carbonate-free. Dowex 50W (X12; 200–400 mesh) was regenerated with NaOH followed by HCl and water. The resin was finally allowed to dry in the atmosphere and kept in a stoppered bottle.

L-Arabitol (Sigma Chemical Co., London) was found to contain traces of galactose and was recrystallized from propan-1-ol before use. α -D-Glucose, α -D-galactose, β -D-mannose and α -L-fucose (AnalaR or reagent grade; British Drug Houses Ltd.) were chromatographically pure and used as supplied.

Methods

Gas-liquid chromatography. The instrument used was a Pye Panchromatograph equipped with a ⁹⁰Sr ionization

detector and a Honeywell-Brown 0-10 mv recorder. The argon supply was dried over molecular sieve Linde 5A (British Drug Houses Ltd.). A Pye standard glass column was used (7 ft. $\times$ $\frac{3}{8}$ in. internal diam.). The column packing was prepared by coating alkali-acid-washed Celite (85-100 mesh) with butane-1,4-diol succinate polyester (Applied Science Laboratory Inc., State College, Conn., U.S.A.) in a concentration of 20% (w/w). The column was packed by means of a vacuum applied to the bottom joint and simultaneous vibration until the required height was reached. The gas-preheating limb of the column was packed with uncoated Celite, and the column was finally conditioned at 200° for 48 hr. before use.

During analysis the following conditions were generally employed: column oven temperature, 142°; flow rate, 25-30 ml./min.; gas inlet pressure, approx. 10-16 lb./in.²; detector oven temperature, 225°; detector voltage, 700v; current range, 10⁻⁸; chart speed, 8 in./hr.

The products of the silylation reaction were injected on to a plug of glass wool contained in a thin glass tube (1 cm. $\times$ 0.4 cm.) and not directly on to the column. The solvents were removed in a stream of argon during about 5 min. and the glass tube was dropped into the top of the column after shutting off the argon supply. The brass cone was quickly replaced and the argon turned on. Peak areas were measured by multiplying the peak height by the width of the peak at half its height.

Hydrolysis of the mucopolysaccharides and preparation of the trimethylsilyl derivatives. For the measurement of the sugars in the hydrolysis products of mucopolysaccharides an internal standard (L-arabitol) was added to the unknown material before hydrolysis. The hydrolysis was then carried out by rendering the mucopolysaccharide solution 0.25N with conc. HCl and adding 10% (w/v) of Dowex 50W (X12; 200-400 mesh). The mixture was heated for 16 hr. (overnight) in screw-capped bottles at 100°. After being cooled, the product was passed down small columns (1 cm. $\times$ 6 cm.) of Dowex 2 (200-400 mesh), the first 2 ml. of eluent was rejected and the next 2.5 ml. collected and pipetted into a flat-bottomed phial equipped with a well-fitting polythene stopper. Control experiments with a standard mixture of the four sugars and L-arabitol in 0.25N-HCl which was analysed by gas-liquid chromatography after passage down the Dowex 2 columns and compared with the same standard mixture of sugars without HCl and before any treatment with resin, showed that the proportions of the various sugars were unaltered. The first 2 ml. of eluent was rejected as it consisted largely of water eluted from the column by the addition of the hydrolysate. As considerable quantities of CO₂ were liberated during the passage of the hydrolysate through the column it was found advantageous to disturb the resin with a piece of wire to dislodge gas locks. A column of the size indicated could handle up to 10 ml. of 0.25N-HCl hydrolysate. The aqueous solution of sugars so obtained was evaporated to dryness in a vacuum desiccator over NaOH pellets and finally silylated with the pyridine-silanes reagent (0.4 ml.). This amount of silylating agent was found to give a maximum yield for up to at least 8 mg. of carbohydrate material. The reaction mixture so obtained was allowed to stand with occasional shaking for about 1 hr. This was ample time for complete reaction, although it was found possible to leave samples much longer without diminution of peak areas. A 10 μ l. Hamilton syringe was used to inject a

suitable amount (usually 5 μ l.) into the glass tube for chromatography.

The pyridine-silanes reagent was prepared by placing pyridine (5 ml.) in a small container and passing a stream of argon or nitrogen over it to displace air. Hexamethyldisilazane (1.5 ml.) and trimethylchlorosilane (1.0 ml.) were added, and the bottle was stoppered immediately. No diminution in peak areas was observed with the reagent up to 2 days old.

The assay for glucose by the glucose oxidase technique (Marks, 1959) and that for fucose by the thioglycollic acid technique (Gibbons, 1955) were carried out on samples of eluates obtained from the Dowex 2 columns.

RESULTS

Pure monosaccharides. D-Glucose, D-galactose, D-mannose and L-fucose each gave a single peak when silylated in the solid state with the pyridine-silanes reagent. The peaks obtained on the chromatogram corresponded to the anomeric form in which the sugar was supplied, this being the α -anomer in every case except mannose. However, when these sugars were obtained by evaporating their aqueous equilibrium solutions and then silylating the solid residue, two major peaks were obtained on the chromatogram, one of which corresponded to the peak of the original solid sugar. It was obvious therefore that the new peak was the other anomeric form of each respective sugar, this therefore being the β -anomer in every case except mannose. In this way it was easy to calculate the retention times of the anomeric forms of the various sugars. With D-galactose and L-fucose there appeared a small third peak on the chromatogram corresponding to the γ -forms of the sugars; these were considered to be furanose or free aldehyde modifications. The peak areas of these γ -modifications were small and amounted to 5% of the areas of the α - and β -peaks of D-galactose and only 1-2% of those of L-fucose. The relative proportions of the peak areas for each sugar were constant and the values agreed well with those obtained by Sweeley *et al.* (1963). The peak areas produced by different amounts (2-10 μ g.) of the trimethylsilyl derivatives of the four sugars were plotted against the amount chromatographed. In each case a linear plot passing through the origin was obtained when the various peaks for each sugar were added together.

The response of the argon detector to the trimethylsilyl derivatives was identical for the three hexose sugars and also for L-fucose and L-arabitol, after due allowance had been made for the lower molecular weight of these derivatives.

The fact that the detector response is identical for such a closely related group of substances is a strong indication that the silylation reaction is quantitative or very nearly so for the four sugars.

This is in agreement with the observation by Perry (1964) in his work on the trimethylsilyl derivatives of *N*-acetyl-amino sugars.

Fig. 1. Gas-liquid chromatography of an aqueous equilibrium mixture of the trimethylsilyl derivatives of D-galactose, D-glucose, D-mannose and L-fucose on butane-1,4-diol succinate polyester at 142° and with 30 ml. of argon/min. The following are the assignments given to the various peaks in order of increasing retention times: 1, γ -fucose; 2, α -fucose; 3, α -mannose; 4 and 5, β -fucose and γ -galactose; 6, α -galactose; 7, α -glucose; 8, β -mannose; 9, β -galactose; 10, β -glucose.

The retention times for a mixture of the four sugars obtained by evaporating an aqueous equilibrium solution are shown in Fig. 1. The order of elution is as follows: γ -fucose, α -fucose, α -mannose, β -fucose and γ -galactose together, α -galactose, α -glucose, β -mannose, β -galactose and finally β -glucose. The γ -galactose and β -fucose peaks are not separated under the conditions described, and it was found that any attempt to bring this about resulted in a poorer separation of one of the other components in the mixture. It was considered, however, that this feature was not very important; it was invariably found that under standardized conditions of hydrolysis and silylation the γ -galactose component was constant and amounted to 5% of the α - and β -peaks. It could therefore be allowed for in the estimation of the β -fucose peak.

The retention time of the internal standard L-arabitol was in the region of the very small γ -fucose component, which was about 1% and considered negligible.

A mixture of the four sugars was subjected to the same hydrolytic conditions as used for the hydrolysis of the mucopolysaccharides and the resulting mixture analysed. The peak areas were found to be unaltered in both total amount and proportions of the various anomers. However, increasing the strength of the hydrochloric acid to 0.5N or 1.0N resulted in decreasing peak areas, suggesting that under these conditions destruction of the sugars would have to be taken into account in any analytical study.

Gastric secretion hydrolysates. A number of gastric secretions from patients under fasting conditions were examined by gas-liquid chromatography after hydrolysis to the component sugars. A small number of salivas were also examined for comparison. In all cases the secretions were dialysed in Visking tubing overnight to remove any low-molecular-weight material before hydrolysis. In

Table 1. Comparison of monosaccharide values for hydrolysates of gastric secretion and saliva by gas-liquid chromatography and manual techniques

All values expressed as μ moles/l.

Sample	D-Galactose	D-Mannose	L-Fucose		D-Glucose	
			Gas-liquid chromatography	Thio-glycollic acid	Gas-liquid chromatography	Glucose oxidase
Gastric secretion 1	4.10	0.70	2.18	2.04	0.88	0.89
Gastric secretion 2	2.00	0.32	1.24	1.30	0.33	0.39
Gastric secretion 3	2.12	0.50	1.42	1.23	0.60	0.60
Gastric secretion 4	4.50	0.54	4.10	4.20	0.90	0.90
Gastric secretion 5	6.86	0.74	6.14	6.10	0.80	0.85
Saliva 1	0.48	0.05	0.34	0.31	—	—
Saliva 2	0.77	0.04	0.77	0.75	—	—
Saliva 3	1.26	0.27	0.86	0.89	—	—

general, the results obtained agreed well with those obtained by paper-chromatographic techniques in this Laboratory (J. Schrager, unpublished work).

A number of typical results for the mucopolysaccharides of gastric secretion and saliva are shown in Table 1. Good agreement was obtained between the values of both glucose and fucose by their respective manual techniques and gas-liquid chromatography.

This is a clear indication of the efficacy of the silylation reaction and the subsequent gas-liquid chromatography when applied to gastric hydrolysates, as there can hardly be any reasonable doubt that D-galactose and D-mannose would react in the same way as the other two sugars whether present as pure anomers or in a gastric hydrolysate.

DISCUSSION

During the preliminary experiments with pure monosaccharides the products of the silylation reaction were injected directly on to the column in the usual manner with a 0-1 μ l. Hamilton syringe. Although some tailing of the pyridine peak did occur even with this quantity, it was not serious and the peak areas could be readily calculated on the pyridine 'tail'. However, with hydrolysates of the mucopolysaccharides it was found desirable to inject up to 5 μ l. of reaction mixture to obtain reasonably sized peaks. This quantity of pyridine caused very considerable tailing, which totally or at best partially blotted out the earlier monosaccharide peaks. In view of this, it was decided to try solid injection, and finally the technique used by Bailey (1964) in his work on steroids was adopted and found to give reproducible results and furthermore to be highly convenient. It was found necessary to remove the solvents with a stream of argon or nitrogen to prevent water vapour reaching the sample. Contamination of the sample, once injected on to the plug of glass wool, resulted in a rising base line that tailed out as far as β -glucose, and was highly undesirable as it rendered calculation of peak areas difficult and liable to inaccuracy.

Incomplete drying of the sample before silylation also gave a similar effect. It was not found necessary to remove all traces of solvent, and in fact any attempt to do this required an excessive length of time. In general 5 min. was adequate to decrease the pyridine peak to a reasonable size. This use of solid injection allowed large samples to be injected if required, thereby increasing the sensitivity and convenience of the gas-liquid-chromatographic technique. At the end of the run the glass tube was readily removed from the column by means of a piece of wire with a small Z bend at the bottom, which caught in the glass plug. On removal the

plug was immediately placed in the argon gas stream to cool, it then being ready for a further injection.

Examination of the chromatogram of the equilibrium mixture of the four sugars shows that, though a number of peaks are not completely resolved, all can be calculated accurately. It seems likely that the use of a longer column would improve the resolution, but this was not considered necessary at the present stage with the materials being analysed. The percentage of the polyester stationary phase was found to be critical for good separation. Too little stationary phase resulted in poor resolution of the α -glucose and α -galactose peaks, whereas too much brought the α -fucose and α -mannose peaks closer together.

L-Arabitol was chosen as an internal standard because it was well separated from the sugar anomers, and it could be added to the mixture before hydrolysis so that any losses or volume changes in this and subsequent steps in the procedure would be automatically accounted for. *myo*Inositol was also tried as an internal standard but unfortunately it was not completely resolved from the β -glucose peak.

The results for gastric-secretion hydrolysates (given in Table 1) indicate the presence of galactose, fucose, mannose and glucose, whereas those for saliva only indicate the presence of the first three monosaccharides; these results are in agreement with those found by paper-chromatographic techniques, but the gas-liquid-chromatographic procedure is obviously advantageous, since it is less time-consuming, considerably more precise and requires far less sample. This last feature is particularly important when examining the eluates of chromatographic columns used for the separation and purification of mucopolysaccharides.

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