THE DETERMINATION OF SUGARS AND AMINO SUGARS IN THE HYDROLYSATES OF MUCOPOLYSACCHARIDES BY GAS-LIQUID CHROMATOGRAPHY

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INTRODUCTION

Recent progress has been made in the application of gas-liquid chromatography to the separation of carbohydrates and related polyhydroxy compounds. As these materials, themselves, are not amenable to gas-liquid chromatography, efforts have been made to convert them to sufficiently volatile derivatives which can be conveniently prepared on a micro scale. At the present time there would seem to be little doubt that the most satisfactory derivatives both from the point of view of performance and ease of preparation are the O-trimethylsilyl ethers of the carbohydrates. A number of papers have appeared describing separations using these derivatives[1-4] and in the latter three cases quantitative results are cited for both neutral and amino sugars. In our previous communication[5] we described the separation and estimation of the neutral monosaccharides found in hydrolysates of human gastric mucopolysaccharides and saliva, and in this work the technique has been extended to include quantitative data on the two amino sugars D-galactosamine and D-glucosamine normally found in the above biological materials.

Materials

Pyridine (AnalaR grade; British Drug Houses Ltd., Poole, Dorset) was dried overnight with BaO and distilled. The distillate was kept over CaH_2 in an amber-coloured bottle.

Trimethylchlorosilane (reagent grade; Hopkin & Williams Ltd., Chadwell Heath, Essex), hexamethyldisilazane (Koch-Light Laboratories Ltd., Colnbrook, Bucks.), and ethyl chloroformate (reagent grade; British Drug Houses Ltd.) were used directly. D-Galactitol, α-D-glucose, α-D-galactose, β-D-mannose, α-L-fucose, D-glucosamine and D-galactosamine (AnalaR or reagent grade; British Drug Houses Ltd.) were chromatographically pure and used as supplied.

"De-Acidite" G (7-9 % crosslinking) 52-100 mesh or 100-200 mesh was treated as follows. The resin was suspended in water, poured into a column and washed with several column volumes of 2 *M* Na_2CO_3 until chloride-free. Washing was continued with water until carbonate-free. Dowex 50 W (X 12,200-400 mesh) was regenerated with NaOH followed by HCl and water.

Preparation of N-carboethoxy D-glucosamine and N-carboethoxy D-galactosamine

D-Glucosamine hydrochloride or D-galactosamine hydrochloride (2 g) was

dissolved in water (20 ml) and to the resulting solution was added ethyl chloroformate (1 ml) and sodium bicarbonate (1.7 g). The reaction mixture was allowed to stand at room temperature overnight. The product was deionized firstly by means of an electrolytic desalter and then by columns of "De-acidite" G (7–9 % crosslinking, 52–100 mesh) and Dowex 50W (X 12, 200–400 mesh). The effluent from the columns was finally freeze-dried to afford the N-carboethoxy amino sugars. Purification was carried out by repeated recrystallisations from propan-1-ol for the D-glucosamine derivative (m.p. 175°) and acetone for the D-galactosamine derivative (m.p. 90°).

METHODS

Gas–liquid chromatography

The separations were obtained on a Pye Series 104 Chromatograph equipped with a hydrogen flame detector, temperature programming unit and a Honeywell-Brown recorder. Column packings were prepared by coating Chromosorb G (AWDMCS, 80–100 mesh) (Johns-Manville Corp., New York), with a solution of Apiezon L and neopentyl glycol adipate (Applied Science Laboratory Inc., State College, Conn., U.S.A.) in benzene to give a final concentration of 2 % (w/w) and 4 % (w/w) respectively, in the final dried packing.

The usual Pye (5 ft. × 3/32 in. I.D.) coiled glass columns were packed with the above material by means of a vacuum applied to the end of the columns, while adding the packing at the other end with simultaneous vibration. Columns were finally conditioned by heating to 245° for 48 h with a nitrogen flow of 100 ml/min and then for a further 24 h at 210° with the same flow rate.

The separations obtained in this report were generally achieved under the following conditions:

Nitrogen carrier gas flow rate 40–45 ml/min; linear temperature programming at 1.5°/min from an initial temperature of 130° to a final temperature of 210°.

Detector conditions: hydrogen flow rate 40–45 ml/min, air flow rate 500 ml/min approx., detector voltage, 45 V.

The amplifier was generally set to an attenuation of 1×10^3 and the chart speed at 10 in/h.

The stainless steel injection port supplied with the Pye 104 series Chromatograph can only be used to inject liquid samples on to the column. However, widening out the narrow hole in the port to one 5/32 in. diameter rendered it very suitable for the solid injection of small glass tubes (1 cm × 0.4 cm) containing plugs of glass wool. The products of the silylation reaction were injected directly on to these glass wool plugs and the volatile solvents rapidly removed by means of a stream of nitrogen. A few minutes later the glass tube was dropped into the top of the column after shutting off the carrier gas and removing the screw cap and septum from the injection port. Immediately following addition, the septum and cap were replaced and the carrier gas supply turned on. At the end of each run the glass tube was removed from the column by means of a piece of thin wire inserted into the glass wool plug. 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

The mucopolysaccharide solutions or suspensions were hydrolysed by rendering them 0.3 *N*, 0.5 *N* and 2 *N* with appropriate amounts of conc. HCl. Usually 5–10 ml

of the material was taken for analysis and the acidified product hydrolysed in a 25 ml screw-capped bottle at 100° for 16 h (overnight). After cooling, a suitable amount of the internal standard (D-galactitol) based on the original volume of mucopolysaccharide solution was added to the material in the screw-capped bottle and the bottle washed out with an equal volume of water in the case of the 0.3 *N* and 0.5 *N* hydrolysates, but with five times its volume of water for the 2 *N* hydrolysates. These solutions were now deacidified by passing down small columns (1 cm × 8 cm) of "De-acidite" G (7–9 % crosslinking 52–100 mesh), the first 4 ml of eluent being rejected and the next 5 ml being collected in a test tube. Occasionally as considerable quantities of CO_2 were liberated during the passage of the hydrolysate down the columns it was sometimes found advantageous to disturb the resin from time to time with a piece of wire to dislodge gas locks. A column of the above size could cater for about 15 ml of 0.3 *N* HCl hydrolysate, and the pH of the eluent was normally between 6.0 and 7.0. Five millilitres of the eluent from the "De-acidite" G column were now treated with 0.3 ml of a freshly prepared saturated solution of $NaHCO_3$ followed by 0.025 ml (2 drops) of ethyl chloroformate; the tube was immediately corked, shaken vigorously and allowed to stand at room temperature for about 1 h. The product was deionized by passing down a small column (1 cm diam.) containing a layer of "De-acidite" G (7–9 % crosslinking, 100–200 mesh) (2 cm height), over which was carefully laid a layer of Dowex 50W (X 12, 200–400 mesh) H^+ form (2 cm height). The first 2.5 ml of eluate was rejected and the next 2 ml collected and pipetted into a flat bottomed phial (20 ml) equipped with a well-fitting polythene stopper. The aqueous solution of sugars and N-carboethoxylated amino 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 reagent 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 at room temperature for about 30 min, this being ample time for complete reaction, although samples could be left much longer without any appreciable diminution of peak areas. A 10 μl Hamilton syringe was used to inject a suitable amount (usually 5–20 μl) on to the glass plug for chromatography.

The pyridine–silanes reagent was prepared by taking the dried pyridine (10 ml) and adding to it hexamethyldisilazane (3 ml) and trimethylchlorosilane (2 ml). The container was stoppered immediately and the liquid swirled gently. This reagent gave satisfactory results for up to at least 3 days, standing at room temperature.

The assay for fucose by the thioglycollic acid technique[6] was carried out on samples of eluates from the "De-acidite" G columns. The determination of total hexosamine by the Elson-Morgan technique modified by Boas[7] was also carried out on these eluates. These samples were also used for the determination of D-glucosamine and D-galactosamine carried out on the Technicon automatic amino acid analyser (Technicon Instrument Co. Ltd., Chertsey, Surrey), with the usual buffer conditions for a 20-hour chromatogram.

RESULTS

Preparation of N-carboethoxy D-*glucosamine, N-carboethoxy* D-*galactosamine and their respective trimethylsilyl ethers for chromatography*

In this series of experiments, D-galactitol was used to follow the N-carbo-

ethoxylation of the two amino sugars in order to determine the optimum reaction conditions for quantitative yields. Standard solutions of the two amino sugars and D-galactitol were prepared by dissolving suitable weighed amounts in water. Aliquots (5 ml) of these standards were taken and reacted with quantities of saturated sodium bicarbonate solution, varying between 0 and 0.4 ml, while the ethyl chloroformate was varied between 0.01 ml–0.04 ml. It was found that except where sodium bicarbonate was absent, the peak areas obtained on subsequent gas–liquid chromatography were not affected within the above limits of the reagents. The reaction time in all cases was one hour at room temperature. In view of the above results, it was decided to use the middle of the range for further experiments, *i.e.* 0.3 ml sodium bicarbonate solution and 0.025 ml ethyl chloroformate. Investigations into the reaction time were now carried out using the above quantities of reagents, and 5 ml of the standard solution. These results are shown in Table I. It will be seen that the N-carboethoxylation was complete for both amino sugars within 15 min, and that the reaction conditions had no deleterious effect on the glucose present, even in the final experiment, which was heated to 80°. In view of these results, one hour at room temperature was generally employed for the reaction.

TABLE I

THE REACTION OF D-GLUCOSAMINE AND D-GALACTOSAMINE WITH ETHYL CHLOROFORMATE IN THE PRESENCE OF SODIUM BICARBONATE

A solution (5 ml) containing D-glucose, D-galactitol, D-glucosamine (1 mM/l) and D-galactosamine (0.5 mM/l) was treated with saturated $NaHCO_3$ (0.3 ml) and Cl·COOEt (0.025 ml). After the appropriate reaction time, the mixture was treated with ion exchange resin as described in the text, and finally silylated (5 μl taken for analysis). Amount recovered in respect to D-galactitol.

Reaction time (min)	D-*Glucose* (mM/l)	D-*Glucosamine* (mM/l)	D-*Galactosamine* (mM/l)
15	0.95	1.01	0.51
30	0.95	0.98	0.50
60	0.99	0.95	0.48
90	0.98	1.03	0.52
90 + 15 at 80°	0.96	0.99	0.50
Mean values	0.97	0.99	0.50

The reactivity of the N-carboethoxy amino sugars with the pyridine–silanes reagent was compared to a typical hexose D-glucose and also with the internal standard D-galactitol. Known weights of the above compounds were dissolved in water and evaporated in the usual manner in a vacuum desiccator. The product was silylated as usual with 0.4 ml reagent, and 5 μl analysed by gas–liquid chromatography at varying time intervals. The areas of the various peaks for the anomers of each sugar were added together, and plotted against the silylation times as shown in Fig. 1. It will be seen that all the components react at approximately the same rate and that the reaction is complete within 10 min.

Anomerisation of N-carboethoxy D-*glucosamine and N-carboethoxy* D-*galactosamine*

When samples of the recrystallised N-carboethoxy amino sugars were reacted with the pyridine–silanes reagent, only one major peak was obtained for each sugar.

However, when these derivatives were prepared in the usual manner from the amino sugars, evaporated and the residue treated with the pyridine–silanes reagent, a second peak with a longer retention time appeared in addition to the original peak, for both sugars. This was considered to be derived from the other anomer of each respective N-carboethoxy amino sugar. For quantitation the areas of both peaks were added together. No attempt was made to correlate each peak to its respective anomeric form (α or β).

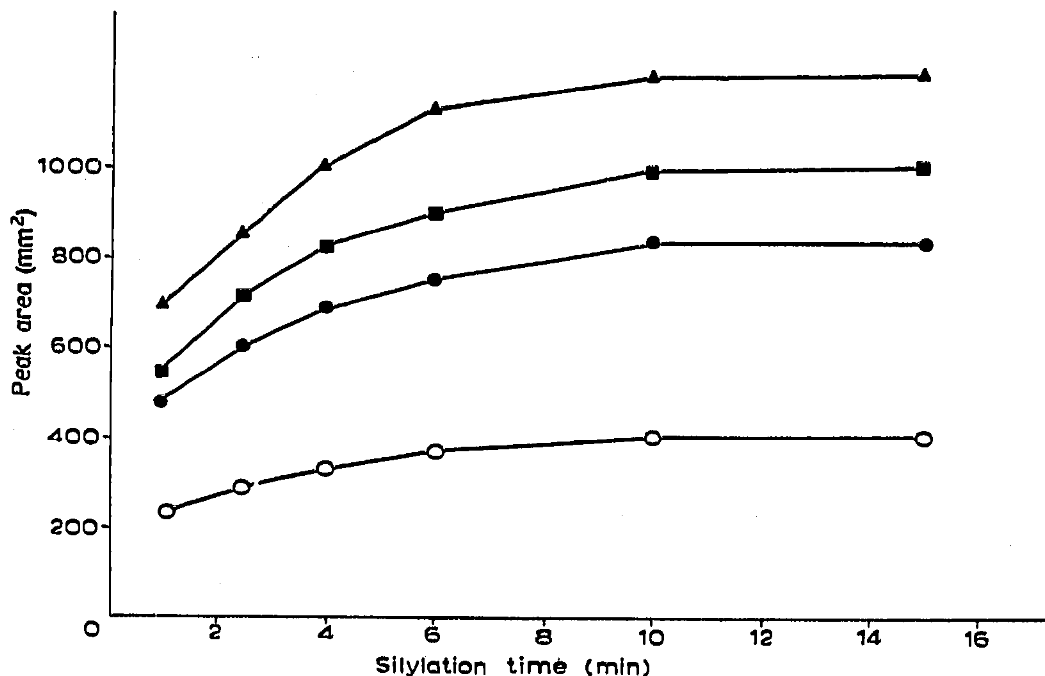

Fig. 1. The reactivity of the N-carboethoxy derivatives of D-glucosamine and D-galactosamine with the pyridine–silanes reagent. A solution (2 ml) containing D-glucose, D-galactitol, N-carboethoxy D-glucosamine (1 mM/l) and N-carboethoxy D-galactosamine (0.5 mM/l) was evaporated and the residue silylated in the usual manner with reagent (0.4 ml). 5 µl was taken for analysis. (▲) D-galactitol; (■) D-glucose; (●) N-carboethoxy D-glucosamine; (○) N-carboethoxy D-galactosamine.

Quantitation of the pure monosaccharides

Due to the anomerisation of the various sugars in water, subsequent chromatography gave two or more peaks from each monosaccharide[5]. The retention times for a mixture of L-fucose, D-galactose, D-mannose, D-glucose, N-carboethoxy D-glucosamine, N-carboethoxy D-galactosamine and the internal standard D-galactitol obtained by evaporating an aqueous equilibrium solution are shown in Fig. 2. The elution order was as follows: γ-fucose, α-fucose, β-fucose, α-mannose, γ-galactose, α-galactose, D-galactitol, α-glucose, β-mannose and β-galactose together, β-glucose, N-carboethoxy galactosamine (1st peak), N-carboethoxy glucosamine (1st peak), N-carboethoxy galactosamine (2nd peak), and finally N-carboethoxy glucosamine (2nd peak). It will be seen that under these conditions the β-mannose and β-galactose peaks failed to separate, so that it became necessary to find the relationship of the β-anomer of D-mannose to its α-anomer. This was determined by preparing a solution of all the sugars with the exception of D-galactose and carrying out the analytical technique including rendering the mixture 0.3 N and 0.5 N with respect to hydrochloric acid.

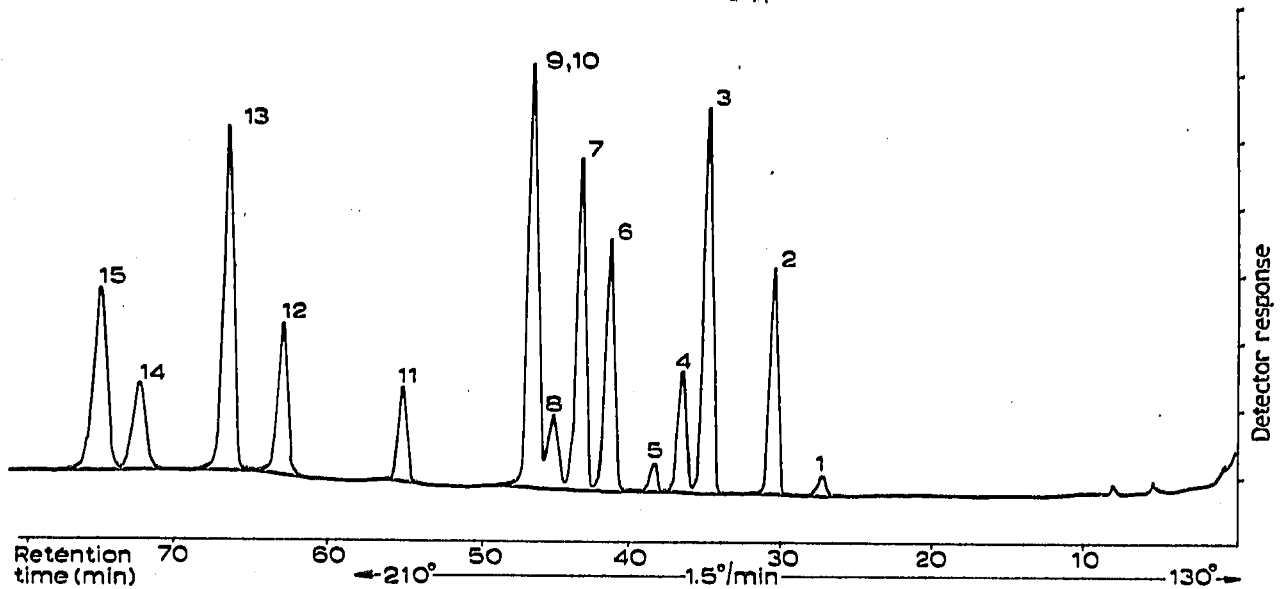

Fig. 2. Gas–liquid chromatography of an aqueous equilibrium mixture of the trimethylsilyl derivatives of D-galactose, D-glucose, D-mannose, L-fucose, N-carboethoxy D-glucosamine and N-carboethoxy D-galactosamine on Apiezon L + neopentyl glycol adipate polyester, with 40 ml N_2/min and linear temperature programming at 1.5°/min. The following are the assignments given to the various peaks in order of increasing retention times: 1 = γ-fucose; 2 = α-fucose; 3 = β-fucose; 4 = α-mannose; 5 = γ-galactose; 6 = α-galactose; 7 = galactitol (std.); 8 = α-glucose; 9 and 10 = β-mannose and β-galactose; 11 = β-glucose; 12 and 14 = N-carboethoxy galactosamine; 13 and 15 = N-carboethoxy glucosamine.

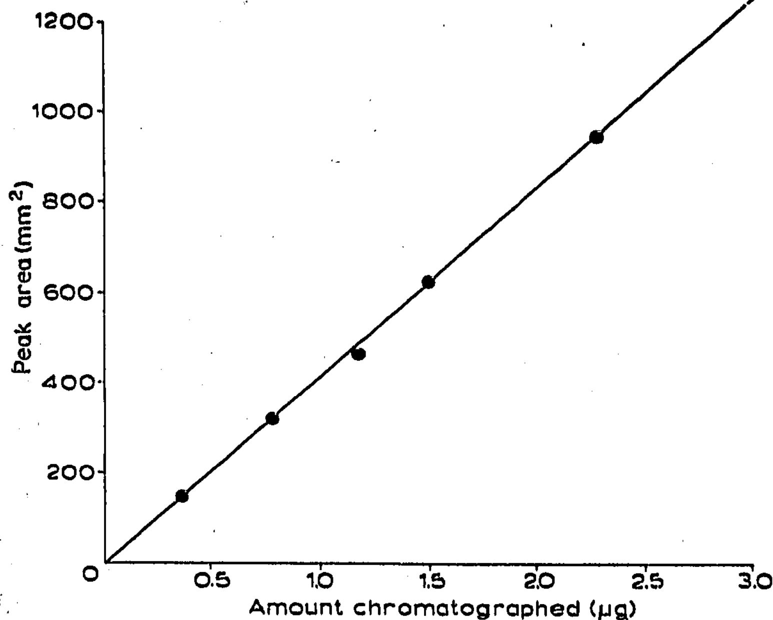

Fig. 3. Proportionality of the gas chromatographic response in terms of peak area to the quantity of N-carboethoxy D-glucosamine silylated for analysis.

Under these conditions it was found that the α-anomer varied between 68–70 % and the β-anomer between 32–30 %. This high level of reproducibility made it possible to calculate the total mannose content merely by measuring the α-mannose peak. The value of the β-mannose peak so calculated was subtracted from the measured β-galactose peak to give the true β-galactose value. The peak areas produced by the trimethylsilyl derivatives of the four sugars, two N-carboethoxy amino sugars and D-galactitol were plotted against the amount chromatographed (0.4–4 μg). In all cases, a linear plot passing through the origin was obtained when the various peaks for each sugar were added together. The plot for N-carboethoxy D-glucosamine is shown in Fig. 3.

By measurement of the slope of the plots obtained, it was possible to calculate the response of the hydrogen flame detector to each sugar derivative and also for the D-galactitol derivative. It was found that the detector response to the three hexose, L-fucose and D-galactitol derivatives was identical for the same weight of each derivative chromatographed. However, although the trimethylsilyl derivatives of the two N-carboethoxy amino sugars each gave an identical response, this response was somewhat less (20 % approx.) than for the other sugars. In this way, factors were determined to correct the observed peak areas for each sugar per mole to that area obtained per mole of D-glucose. These were as follows: D-glucose, D-galactose, D-mannose, 1.00; L-fucose, 1.20; D-galactitol, 0.88; D-glucosamine and D-galactosamine, 1.24.

Quantitative analysis of pure monosaccharides by the gas chromatographic technique

A mixture of the four sugars and two amino sugars was prepared in the following concentrations: L-fucose, D-galactose and D-glucosamine, 1 mM/l; D-galactosamine, 0.5 mM/l; and D-mannose and D-glucose, 0.25 mM/l. This solution (5 ml aliquots) was now treated with the required amount of conc. hydrochloric acid and internal standard (0.2 ml of a 10 mM/l solution) and subjected to the full analytical technique except that the overnight hydrolysis at 100° was omitted. The results obtained are reproduced in Table II. It will be seen that excellent agreement was obtained for every sugar. The above series of experiments was repeated except that the hydrolysis step at 100° overnight was now included (Table III). It will be seen that the neutral monosaccharides were recovered completely at 0.3 N but at 0.5 N slight destruction occurs, leading to somewhat low recoveries of L-fucose and D-galactose. For the 2N conditions, this loss was considerable. The amino sugars were recovered unchanged under all these conditions.

Comparison of the gas–liquid chromatography technique with the Technicon Auto-Analyser and the manual Elson-Morgan method

A series of standard solutions of D-glucosamine and D-galactosamine were made up and their concentration determined by each of the above methods. In the auto-analyser technique, the buffer conditions were those normally employed for the determination of amino acids and nor-leucine was used as an internal standard. The Elson-Morgan technique measured total hexosamine[6]. The gas–liquid chromatography was carried out as previously described with D-galactitol as the internal standard. The results are shown in Table IV. It will be seen that good agreement was obtained between the three techniques.

TABLE II

QUANTITATIVE ANALYSIS OF A MIXTURE OF PURE MONOSACCHARIDES BY THE GAS CHROMATOGRAPHIC TECHNIQUE

A standard solution of the six sugars was prepared, acidified with the appropriate quantity of HCl and subjected to the usual analytical procedure as described in the text. All values are expressed in m*M*/l.

Hydrolysis normality	D-*Galactose* (*1.00 mM/l*)	L-*Fucose* (*1.00 mM/l*)	D-*Mannose* (*0.250 mM/l*)	D-*Glucose* (*0.250 mM/l*)	D-*Glucosamine* (*1.00 mM/l*)	D-*Galactosamine* (*0.500 mM/l*)
0.3	0.97	1.02	0.240	0.240	1.01	0.500
0.3	1.01	0.99	0.240	0.234	1.00	0.500
0.3	0.99	1.04	0.247	0.255	1.01	0.514
0.3	0.95	1.01	0.245	0.239	1.02	0.494
0.5	0.98	1.02	0.252	0.240	1.03	0.530
0.5	1.06	1.00	0.250	0.248	1.02	0.495
0.5	0.97	1.00	0.255	0.243	0.99	0.491
Mean values	0.99	1.01	0.247	0.245	1.01	0.504
2	1.02	1.04	0.240	0.253	1.00	0.500
2	1.01	1.04	0.242	0.236	1.04	0.500
2	0.98	1.00	0.245	0.248	1.00	0.491
Mean values	1.00	1.03	0.243	0.246	1.01	0.494

TABLE III

THE EFFECT OF INCREASING ACID STRENGTH ON THE RECOVERIES OF PURE MONOSACCHARIDES

A standard solution of the six sugars was prepared, hydrolysed at 100° overnight with the appropriate quantity of HCl and subjected to the usual analytical procedure as described in the text. All values are expressed in m*M*/l.

Hydrolysis normality	D-*Galactose* (*1.00 mM/l*)	L-*Fucose* (*1.00 mM/l*)	D-*Mannose* (*0.250 mM/l*)	D-*Glucose* (*0.250 mM/l*)	D-*Glucosamine* (*1.00 mM/l*)	D-*Galactosamine* (*0.500 mM/l*)
0.3	1.01	1.00	0.236	0.240	1.00	0.482
0.3	0.98	1.00	0.240	0.260	0.98	0.471
0.3	0.98	0.97	0.253	0.255	1.01	0.483
0.3	0.97	0.99	0.244	0.260	1.02	0.496
Mean values	0.98	0.99	0.244	0.254	1.00	0.483
0.5	0.89	0.96	0.243	0.245	1.00	0.493
0.5	0.96	0.95	0.236	0.258	0.98	0.480
0.5	0.94	0.97	0.244	0.250	1.00	0.495
0.5	0.94	0.98	0.249	0.253	1.02	0.500
Mean values	0.93	0.96	0.243	0.251	1.00	0.492
2	0.72	0.66	0.122	0.180	1.04	0.495
2	0.62	0.60	0.144	0.166	1.03	0.512
2	0.69	0.43	0.102	0.178	1.03	0.472
2	0.72	0.73	0.183	0.167	1.01	0.492
Mean values	0.64	0.63	0.138	0.173	1.03	0.493

TABLE IV

A COMPARISON OF THE GAS–LIQUID CHROMATOGRAPHIC, TECHNICON AUTO ANALYSER AND ELSON-MORGAN TECHNIQUES FOR THE DETERMINATION OF HEXOSAMINES

GNH_2 = D-glucosamine; Gal NH_2 = D-galactosamine. The analytical procedures are described in the text.

Amount taken (mM/l)		*Amount recovered (mM/l)*				
GNH₂	*Gal NH₂*	*Technicon Auto Analyser*		*Gas–liquid chromatography*		*Elson-Morgan*
		GNH₂	*Gal NH₂*	*GNH₂*	*Gal NH₂*	*Total hexosamine*
0.20	0.10	0.198	0.091	0.201	0.090	0.29
0.30	0.15	0.281	0.146	0.320	0.158	0.50
0.36	0.18	0.336	0.178	0.340	0.175	0.57
0.40	0.20	0.391	0.179	0.440	0.207	0.61
0.60	0.30	0.567	0.290	0.620	0.316	0.93

The release of monosaccharides from gastric secretion during acidic hydrolysis

In this series of experiments, gastric secretion obtained from patients under fasting conditions was subjected to hydrolysis under varying normalities of HCl at 100° overnight. These results are shown in Table V. It will be seen that the sugars are generally released in the following order: L-fucose first, then the three hexoses at

TABLE V

THE RELEASE OF MONOSACCHARIDES FROM GASTRIC SECRETIONS DURING ACIDIC HYDROLYSIS

All gastric secretions were hydrolysed overnight at 100° with the appropriate quantity of HCl. The analytical procedure was as described in the text. All values are expressed as m*M*/l.

Hydrolysis normality	*D-Galactose*	*L-Fucose*		*D-Mannose*	*D-Glucose*	*D-Galactosamine*	*D-Glucosamine*	*Total hexosamine Elson-Morgan*
		Gas chromatography	*Thioglycollic acid*					
Gastric secretion (1)								
0.1	0.310	0.217	0.23	0.074	0.077	0.120	0.253	0.48
0.3	0.315	0.230	0.22	0.074	0.074	0.150	0.303	0.47
0.5	0.300	0.217	0.22	0.074	0.090	0.133	0.288	0.47
1.0	0.220	0.110	0.13	0.060	0.070	0.130	0.270	0.47
2.0	0.123	0.070	0.10	0.030	0.043	0.135	0.280	0.42
3.0	—	—	—	—	—	0.145	0.270	0.42
Gastric secretion (2)								
0.05	0.64	0.274	0.34	0.114	0.230	0.099	0.420	0.82
0.1	0.78	0.371	0.33	0.171	0.291	0.142	0.560	0.86
0.3	0.75	0.355	0.35	0.150	0.290	0.155	0.631	0.85
0.5	0.74	0.355	0.35	0.150	0.293	0.165	0.641	0.85
1	0.67	0.330	0.34	0.130	0.243	0.167	0.625	0.84
2	0.24	0.074	0.09	0.151	0.103	0.170	0.624	0.82
3	—	—	—	—	—	0.180	0.647	0.80
Gastric secretion (3)								
0.3	0.923	0.403	0.39	0.135	0.150	0.253	0.916	1.26
0.5	1.10	0.421	0.39	0.160	0.253	0.276	1.03	1.48
2	—	—	—	—	—	0.353	1.05	1.48
3	—	—	—	—	—	0.360	1.00	1.48

approximately the same rate and finally D-glucosamine and D-galactosamine. These and other experiments not shown in Table V, led to the conclusion that in general L-fucose was released completely at 0.3 N HCl, and very often the three hexoses were also released. At 0.5 N HCl, L-fucose was showing a certain amount of destruction as also were the hexoses in some cases, although in general only slightly. D-glucosamine usually reached a maximum value at this normality of acid, and in certain cases, D-galactosamine also. In many instances, however, it was found necessary to resort to 2 N HCl for complete release of D-galactosamine. Under these conditions all the neutral sugars suffered destruction, making their analytical values useless. In view of these results, it was decided to standardise 0.3 N, 0.5 N and 2 N HCl hydrolysis conditions for all material under investigation, to obtain a complete record for every sugar. Where only amino sugars were being investigated, however, it was necessary to carry out the 2 N HCl hydrolysis only. The reproducibility of a series of hydrolyses on the gastric secretion obtained from one individual is shown in Table VI. The agreement obtained under any one set of conditions was excellent, although the values of the various sugars vary under different hydrolytic conditions, due to destruction on the one hand and further sugar release on the other.

It should be noted that in several of the tables, values for L-fucose as determined by the thioglycollic acid method, and total hexosamine by the Elson-Morgan technique have been included for comparison with the gas-liquid chromatography figures.

TABLE VI

QUANTITATIVE ANALYSIS OF MONOSACCHARIDES OBTAINED BY THE HYDROLYSIS OF A POOLED GASTRIC SECRETION

The gastric secretion was hydrolysed overnight at 100° with the appropriate quantity of HCl. Aliquots were taken for analysis by the procedures described in the text. All values are expressed in mM/l.

Hydrolysis normality	D-*Galactose*	L-*Fucose*	D-*Mannose*	D-*Glucose*	D-*Glucosamine*	D-*Galactosamine*
0.3	0.664	0.562	0.137	0.121	0.594	0.107
0.3	0.645	0.553	0.134	0.121	0.564	0.117
0.3	0.621	0.560	0.135	0.117	0.560	0.112
0.3	0.660	0.570	0.135	0.122	0.560	0.111
Mean values	0.650	0.562	0.135	0.120	0.570	0.112
0.5	0.585	0.492	0.136	0.110	0.643	0.154
0.5	0.615	0.455	0.130	0.103	0.664	0.160
0.5	0.600	0.500	0.131	0.105	0.680	0.162
0.5	0.630	0.502	0.133	0.111	0.674	0.160
Mean values	0.607	0.488	0.132	0.108	0.665	0.159
2	—	—	—	—	0.630	0.162
2	—	—	—	—	0.690	0.167
2	—	—	—	—	0.655	0.155
2	—	—	—	—	0.660	0.162
2	—	—	—	—	0.640	0.165
2	—	—	—	—	0.630	0.158
Mean values	—	—	—	—	0.651	0.162

Generally good agreement was obtained. A typical chromatogram for a gastric secretion hydrolysed with 0.5 *N* HCl is reproduced in Fig. 4.

Investigations undertaken with saliva showed that its component sugars were released under very similar conditions to the gastric mucopolysaccharides and the same hydrolysis conditions were therefore standardised for experiments on this material.

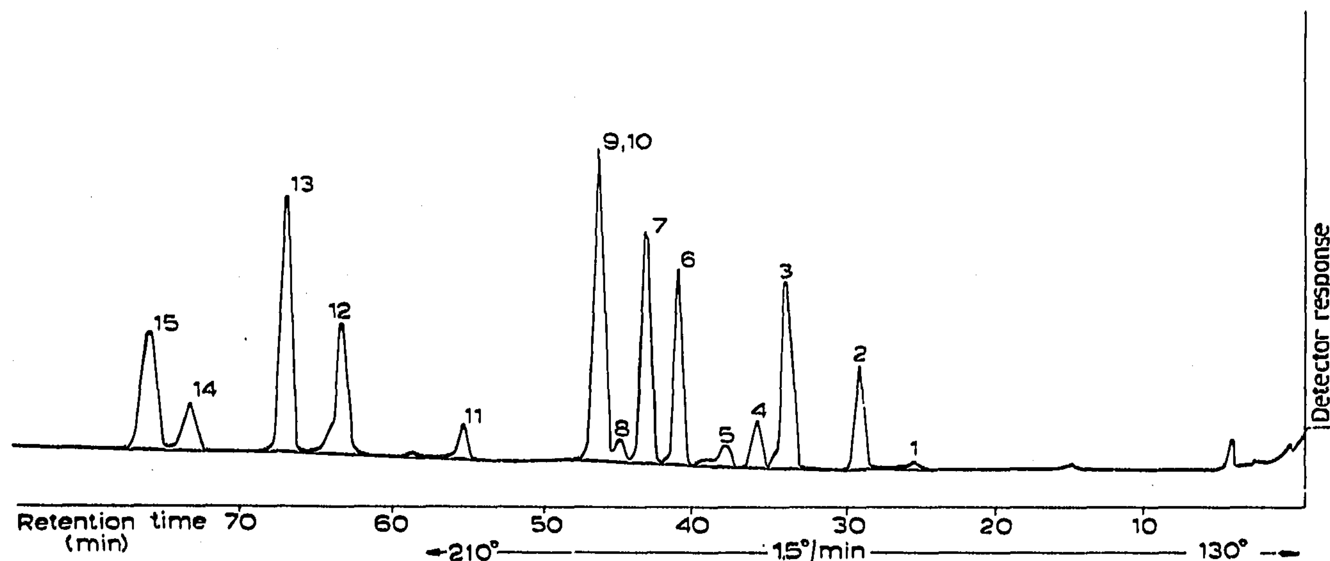

Fig. 4. A typical gas-liquid chromatogram of a gastric secretion hydrolysed with 0.5 *N* HCl and analysed as described in the text. The chromatogram was obtained under the same conditions as given in the legend of Fig. 2, and similar assignments have been given to the peaks.

DISCUSSION

Previous investigators[2,4] working on the estimation of amino sugars by gas-liquid chromatography, have commonly used the trimethylsilyl ethers of the N-acetyl amino sugars. During preliminary work, the trimethylsilyl derivatives of both the free and N-acetylated amino sugars were examined. It was found that the derivatives of the free amino sugars gave much smaller peaks than would have been expected, and that these peaks had a similar retention time to α-galactose. It was therefore considered that these derivatives did not merit further investigation. The trimethylsilyl ethers of the N-acetyl amino sugars gave only one peak when prepared from each respective crystalline solid N-acetyl amino sugar, but when prepared by acetylation of the amino sugars directly, two major peaks appeared and also sometimes a third minor peak in both cases. Attempts to quantitate the derivatives of the two N-acetyl amino sugars were only partially successful as the reproducibility from sample to sample was poor. This appeared to be due to decomposition or absorption on the column, with consequent reduction in the expected peak areas. BISHOP, COOPER AND MURRAY[8] mention the decomposition of fully acetylated amino sugars on gas chromatography columns, and it may well be that similar reactions occur with these N-acetyl-O-trimethylsilyl derivatives. RICHEY, RICHEY JR. AND SCHRAER[3] carried out quantitative experiments on both N-acetyl glucosamine and N-acetyl galactosamine and also apparently suffered from column absorption effects, necessitating rigorously controlled conditions

for successful quantitative analysis. In view of these preliminary experiments with N-acetylated amino sugars, it was decided to investigate the possibility of using other derivatives for gas–liquid chromatography, namely the trimethylsilyl ethers of the N-carboethoxy amino sugars. The experiments carried out with these derivatives showed that they did not suffer any absorption or apparent decomposition on the columns used, and as expected, only two peaks were obtained from each of the two amino sugars. The detector response for the two derivatives was also identical. These findings indicate the superiority of these derivatives over the N-acetyl-O-trimethylsilyl compounds; in addition, their preparation is easier and does not require the use of an internal standard to follow the N-carboethoxylation step in the procedure. Solid injection was again adopted[5,9] in order to allow the use of large volumes of sample (10 μl or more). This technique had the further advantage of eliminating column contamination, it only being necessary to replace the glass wool plugs occasionally when they became very dirty. In our previous communication[5] it was noted that contamination of the sample with water vapour resulted in a highly objectionable rising base line which often stretched as far as β-glucose; it has been found, however, that the hydrogen flame detector does not show this effect, so that complete exclusion of water from the sample and pyridine–silanes reagent is not now of paramount importance. Furthermore, the flame detector has been found preferable as it does not suffer from contamination and response variations to the same extent as the argon detector when used with these silicon-containing derivatives. The flame detector has now been in daily use for over six months and has given no trouble apart from requiring occasional jet cleaning, indicating its suitability for the analysis of derivatives of the type under investigation.

It was found that the separation of the various sugar anomers could be controlled by slight variations in the ratio of the Apiezon L to the neopentyl glycol adipate. It was felt that this was a more convenient technique than searching for one stationary phase of a suitable polarity to bring about the required separations. It might be considered that the estimation of D-mannose using only the α-mannose peak is not completely satisfactory and indeed this would probably be the case where the ratio of D-mannose to D-galactose is high; in the case of gastric secretion and saliva, however, where the D-mannose content is generally comparatively low, the estimation of the α-mannose peak only gives highly reproducible results for both D-galactose and D-mannose.

It was found that the response per unit mass of the hydrogen flame detector to the various trimethylsilyl derivatives of the four neutral sugars and for D-galactitol was identical, thereby indicating that the trimethylsilylation reaction was quantitative. In the case of the two N-carboethoxy amino sugars, however, the response per unit mass was about 20 % less than might have been expected, presumably due to the different structural group present in these compounds[10], as no evidence for column absorption tendencies was noted.

The results show that for the pure amino sugars, excellent agreement was obtained between the Elson-Morgan technique and that involving the Technicon Auto-Analyser, but obviously that the gas chromatographic technique is much quicker than the Auto-Analyser and superior to the Elson-Morgan technique, which only gives the total hexosamine value. It should be noted that the total hexosamine values for gastric secretion determined by the Elson-Morgan method do not always

agree with the gas chromatography method and, in general, are often significantly higher, particularly where complete acidic hydrolysis has not been achieved. This is considered to be due to two factors, firstly that the colour reaction is still complete even though the amino sugars are not completely released and secondly that the neutral sugars and amino acids present in the hydrolysates have combined to form a chromogen which contributes to the Elson-Morgan reading[11,12].

It is apparent, therefore, that gas chromatography marks a notable advance over the above technique in that it can be conveniently used to follow the release of each amino sugar in the hydrolysates of mucopolysaccharides, and also, of course, for the concomitant release of any neutral sugars present as well, thereby affording information on structural positions of monosaccharides present in the mucopolysaccharides. The experiments carried out with gastric secretions to determine the optimum hydrolytic conditions for the various sugars show the importance of these conditions to obtain complete hydrolysis without significant loss of the more labile neutral sugars. It was found impossible to arrive at one particular acid strength to release all the sugars without some destruction of the more labile ones and furthermore, gastric secretions tended to vary in their resistance to hydrolysis; for this reason, it was necessary to standardise three different hydrolytic conditions to obtain full analytical data for the four neutral and two amino sugars normally present in gastric secretions. It should be mentioned that the gas chromatographic technique for analysing gastric secretions has now been in daily use for some six months and have given virtually no trouble during this time, thereby indicating its suitability for the analysis of these materials on a routine basis.

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SUMMARY

Conditions are described for the simultaneous gas chromatographic determinations of L-fucose, D-mannose, D-galactose, D-glucose, D-glucosamine and D-galactosamine.

The technique involves the carboethoxylation of the amino group present in the amino sugars followed by trimethylsilylation of the hydroxyl groups. The neutral monosaccharides are trimethylsilylated directly in the usual manner and the final reaction mixture analysed by gas–liquid chromatography on a single column with temperature programming.

The release of the component monosaccharides from human gastric mucopolysaccharides and saliva with increasing acidity was followed by gas–liquid chromatographic determinations on the hydrolysates.

Quantitative data on standard mixtures of amino sugars showed good agreement between this technique and others, involving either the Technicon Auto-Analyser, or the manual Elson-Morgan method.

The gas chromatographic technique is considered to be superior to either of the

above methods of analysis, particularly with regard to sensitivity, speed and convenience.

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