DETERMINATION OF GLUCOSAMINE AND GALACTOSAMINE BY GAS CHROMATOGRAPHY

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Although methods have been developed for the determination of the total hexosamine content in mucopolysaccharides[1-3], the determination of glucosamine and galactosamine separately has been tedious or inaccurate[4]. Recent developments in gas chromatographic techniques for the analysis of polar compounds offer, however, new possibilities for the characterization of the amino sugars in hydrolyzates of mucopolysaccharides.

According to PERRY[5] the gas chromatographic analysis of glucosamine and galactosamine can be carried out using their N-acetylated trimethylsilyl (TMS) derivatives. The gas chromatographic behaviour of N-acetylated TMS-hexosamines has also been studied by RICHEY *et al.*[6] and SWEELEY *et al.*[7,8].

When non-polar stationary phases, such as SE-52 and SE-30, are employed, no N-acetylation is needed to obtain separation of TMS-hexosamines[7,8]. In the present paper conditions for trimethylsilylation of *non-acetylated* hexosamines as well as the gas chromatography of the TMS-derivatives are investigated. The results are applied to the quantitative determination of glucosamine and galactosamine of mucopolysaccharide hydrolyzates.

MATERIALS AND METHODS

Materials

D-Glucosamine hydrochloride, specific rotation $+72.8°$ ($c = 1$, water), homogeneous in paper chromatography, and D-galactosamine hydrochloride, specific rotation $+97.2°$, homogeneous in paper chromatography, were obtained from Mann Research Laboratories, Inc., New York, N.Y. D(+)-Glucose, analytical reagent grade (B.D.H., Poole, Great Britain), galactose, *puriss.* (E. Merck A.G., Darmstadt), sorbitol (E. Merck A.G., Darmstadt), hexamethyldisilazane, *purum* 98 % (Fluka A.G., Buchs SG), trimethylchlorosilane, *puriss.* 99 % (Fluka A.G.), and pyridine, reagent grade (J. T. Baker Chemical Co., Phillipsburg, N.J.), were used without further purification.

Extraction and purification of mucopolysaccharides

Acetone defatted samples of various connective tissues (bovine skin, human umbilical cord, bovine aorta and laryngeal cartilage) were hydrolyzed with papain as described by SCHILLER *et al.*[9]. The material soluble in 10 % (w/v) trichloroacetic acid was dialyzed against distilled water at 4°.

The mucopolysaccharides were precipitated at 4° with 4 volumes of ethanol which contained 0.5 % sodium acetate and the precipitate dissolved in water. Samples from the aorta and cartilage were purified further by a precipitation with excess cetylpyridinium chloride (CPC)[10]. The CPC-complexes were dissolved in 3 N $MgCl_2$ and the acid mucopolysaccharides precipitated again with ethanol.

The papain-liberated mucopolysaccharides of the human umbilical cord were dissolved in 0.3 N $MgCl_2$, and a fraction containing mainly chondroitin sulphates was precipitated with excess CPC (fraction I). Hyaluronic acid was recovered from the supernatant by a precipitation with ethanol (fraction II).

The mucopolysaccharides were hydrolyzed in 2 N hydrochloric acid in sealed tubes at 103° for 17 h. The separation of hexosamines from other carbohydrates was accomplished by using columns of Dowex-50 as described by BOAS[11].

Trimethylsilylation

The sample (0.01–1 mg in 1–2 ml aqueous solution) to be analyzed was mixed with an internal standard (sorbitol 0.01–1 mg) in a conical centrifuge tube. The solvent was evaporated in a stream of nitrogen and the material dried further in a vacuum oven at 50° for 15 min. Pyridine (0.07 ml), hexamethyldisilazane (0.02 ml) and trimethylchlorosilane (0.01 ml) were added successively, the solution was gently shaken for 30 sec and the mixture left to stand at room temperature for 20 min.

Gas chromatography

A Barber-Colman M-10 chromatograph equipped with a flame ionization detector was employed with nitrogen as carrier gas. The columns were 6 ft × 4 mm glass U-tubes, inactivated conventionally with trimethylchlorosilane and hexamethyldisilazane. Two stationary phases, QF-1 (1 %, w/w) and SE-30 (3 %, w/w) on 100–140 mesh siliconized Gas-Chrom P (Applied Science Laboratories) were used in the experiments.

Injections into the gas chromatograph (0.5–2 μl) were made directly from the reaction mixture with a 10 μl Hamilton microsyringe. For quantitative analyses the peaks were cut off from the chromatograms and weighed.

Statistical methods

The standard deviation (s) for a series of N experiments was calculated from the formula:

$$s = \sqrt{\frac{S(d^2)}{N-1}},$$

where $S(d^2)$ is the sum of squared deviations from the mean.

Precision was expressed as standard deviation (s) calculated from the formula:

$$s = \sqrt{\frac{S(d^2)}{2N}}$$

where $S(d^2)$ is the sum of squared differences between the results of duplicate analyses and N is the number of duplicate determinations[12].

RESULTS AND DISCUSSION

Qualitative analysis

On 1 % QF-1 at 144°, both TMS-glucosamine and TMS-galactosamine give rise to two separate peaks, but overlapping occurs when the hexosamines are chromatographed simultaneously (see Table I).

TABLE I

RETENTION TIMES OF TRIMETHYLSILYL DERIVATIVES OF CARBOHYDRATES

All retention times are relative to α-glucose. Each figure indicates a separate peak.

	Stationary phase QF-1			*Stationary phase SE-30*					
	144°			*170°*			*205°*		
Glucosamine	1.18	1.29	—	0.90	1.13	—	0.93	1.10	—
Galactosamine	1.04	1.20	—	0.79	—	—	0.81	—	—
Glucose	1.00	1.59	—	1.00	1.55	—	1.00	1.32	—
Galactose	0.81	0.98	1.15	0.75	0.89	1.07	0.75	0.90	1.04
Sorbitol	—	—	—	1.32	—	—	1.25	—	—

On 3 % SE-30 at 205° TMS-glucosamine produces two peaks while only one peak is obtained from TMS-galactosamine (Table I, Fig. 1). The peaks of TMS-hexosamines are symmetrical and well separated from one another. The ratio of the two TMS-glucosamine peak areas is constant under standard conditions when aqueous solvents are used in the preparation of the samples.

As natural mucopolysaccharide preparations often contain hexoses, which may

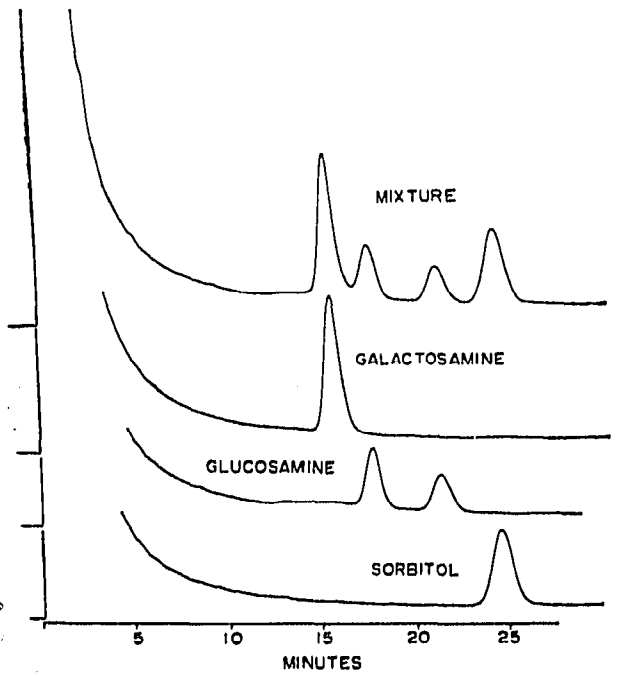

Fig. 1. Gas chromatographic patterns of glucosamine (100 μg), galactosamine (100 μg) and sorbitol (50 μg) as their trimethylsilyl derivatives. Stationary phase 3 % SE-30 at 205°.

interfere in the gas chromatography of hexosamines, the latter have to be purified with Dowex-50. Fig. 2 shows gas chromatography of the purified hexosamine fraction from bovine aorta.

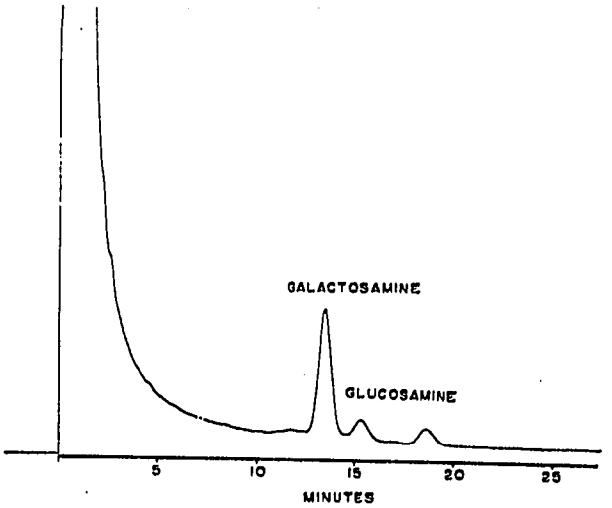

Fig. 2. Gas chromatographic pattern of hexosamines in the hydrolyzed mucopolysaccharide fraction (200 μg) of bovine aorta. Stationary phase 3 % SE-30 at 205°.

Quantitative analysis

SE-30 (3 %, w/w) at 205° was employed in the quantitative determinations. Sorbitol was selected as internal standard, because its TMS-derivative was well separated on SE-30 from the corresponding derivatives of all common hexoses and hexosamines.

The silylation reaction was found to proceed rapidly and quantitatively when the silylating reagents (hexamethyldisilazane and trimethylchlorosilane) were present in sufficient excess in the reaction mixture. The reaction was complete in 15–20 min. When the ratio of hexosamines to the silylating reagents was greater than 1:10, formation of the TMS-derivatives was incomplete resulting in smaller peak areas and in the appearance of extra peaks in the chromatograms. Small changes in the relative proportions of the reagents in the silylation mixture did not affect the results.

TABLE II

DETERMINATION OF THE RELATIVE RESPONSE OF THE FLAME IONIZATION DETECTOR TO TMS-GLUCOSAMINE, TMS-GALACTOSAMINE AND TMS-SORBITOL

Aliquots of 1 μl (equal to 1 % of reaction mixture) were injected into the column. The values were calculated from 5 replicate estimations. Stationary phase 3 % (w/w) SE-30 at 205°.

Amount in the reaction mixture		*Relative response*	
Hexosamine (μg)	*Sorbitol* (μg)	*Glucosamine/ sorbitol*	*Galactosamine/ sorbitol*
100	12.5	0.570 ± 7.2 %	0.725 ± 7.9 %
100	50	0.550 ± 8.2 %	0.720 ± 5.2 %
100	200	0.530 ± 5.7 %	0.685 ± 4.7 %

Because of the difference in response of the flame ionization detector for TMS-hexosamines and sorbitol, it is necessary to determine calibration coefficients for the peak areas. Reproducibility of the ratio of peak areas was satisfactory as shown in Table II. The ratio was not affected by sample size for values above 10 μg (equal to 0.1 μg in the injection mixture). The smallest detectable amount of hexosamine was of the order of 1 μg (equal to 0.01 μg in the injection mixture).

Reproducibility of the method was determined from duplicate analyses of five biological samples (Table III). As the samples were divided into two equal portions after purification with Dowex-50, the errors due to isolation, hydrolysis and column purification are not encountered in the data of Table III. Standard deviation within the duplicates was 3.7 % for glucosamine and 6.2 % for galactosamine (the deviations were expressed as % of total hexosamine in each sample). The reproducibility of the ratio of the two hexosamines gave still better precision.

TABLE III

DETERMINATION OF GLUCOSAMINE AND GALACTOSAMINE IN MUCOPOLYSACCHARIDE HYDROLYZATES

Preparation of samples as described in the text. Stationary phase 3 % SE-30 at 205°. A and B refer to duplicate analyses.

Source of mucopolysaccharide		*Glucosamine*		*Galactosamine*	
		μg	%*	μg	%*
Bovine laryngeal cartilage	A	21.7	7.8	257.8	92.0
	B	30.8	9.9	277.4	90.1
Bovine aorta	A	64.9	28.3	164.2	71.7
	B	74.5	30.4	170.6	69.6
Bovine skin	A	13.9	28.4	35.0	71.6
	B	16.7	27.2	45.0	72.8
Umbilical cord (fraction I)	A	36.6	9.4	354.8	90.6
	B	32.2	9.3	313.9	90.7
Umbilical cord (fraction II)	A	259.8	88.1	35.1	11.9
	B	239.0	87.8	33.3	12.2

* % of total hexosamine.

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SUMMARY

A gas chromatographic method for simultaneous analysis and quantitative determination of glucosamine and galactosamine is described. Hexosamines were isolated from mucopolysaccharide hydrolyzates with Dowex-50 and converted to trimethylsilyl derivatives with trimethylchlorosilane and hexamethyldisilazane in

pyridine solution at room temperature. A non-polar stationary phase SE-30 was employed in quantitative determinations. Sorbitol was used as internal standard.

Duplicate analyses on biological samples are described. The minimum detectable amount of hexosamine was about 0.01 μg. The standard deviation of duplicates was 3.7 % for glucosamine and 6.2 % for galactosamine. The standard deviation of replicates was about 8 % or less.

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