GAS-LIQUID CHROMATOGRAPHY OF HEXOSAMINES

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1. Introduction

The analysis of hexosamines is usually performed by colorimetric methods [1,2] that do not differentiate between individual amino sugars. It is therefore necessary to separate the hexosamines prior to analysis [3–5]; this is laborious and requires large quantities of materials.

The analysis of carbohydrates, as their trimethylsilyl (TMS) derivatives, by gas-liquid chromatography (GLC) has proved to be a rapid and sensitive technique [6]. However, hexosamine hydrochlorides do not readily give volatile TMS derivatives [7] except if *N*-acetylated prior to silylation [7,8] or if directly trimethylsilylated, as in the method of Radhakrishnamurphy, Dalferes and Berenson [9].

In this study hexosamines were trimethylsilylated using bis(trimethylsilyl)trifluoroacetamide (BSTFA) in dimethylacetamide. BSTFA has recently been used very successfully in the formation of *N*-TMS amino acid TMS esters and its advantages are well documented [10].

2. Materials and methods

2.1. *Materials*

The D-hexosamine hydrochlorides and BSTFA (Sigma), and phenanthrene (BDH) were used without further purification.

Dimethylacetamide (BDH) was stored over anhydrous $CaSO_4$.

The cartilage proteoglycan used for testing this method was prepared from bovine nasal septa by the procedure of Malawista and Schubert [11], followed by fractionation according to Gerber, Franklin and Schubert [12].

A Pye 104 model 64 gas chromatograph with a single column was used for the chromatography.

2.2. *Chromatographic conditions*

Column: 5 foot glass; 3% Apiezon L on Diatomite CQ, 100–120 mesh
Column temperature: 175°, isothermal
Detector temperature: 200°
Flash heater: 220°
Attenuation: 2×10^2
Carrier flow, argon: 45 ml/min
Air (to detector): 600 ml/min
Hydrogen (to detector): 40 ml/min
Chart speed: 5 mm/min

2.3. *Derivatization procedure*

Solutions, containing from 10–100 μg of hexosamines were evaporated to dryness in small Teflon-capped tubes. To each sample 0.1 ml BSTFA reagent was added and the tubes were heated at 100° for 10 min. (The BSTFA reagent consisted of 25% BSTFA in dimethylacetamide with phenanthrene, as internal standard, at a concentration of 40 μg/0.1 ml). The tube was then cooled to room temperature and 1 μl of the sample was injected into the column.

2.4. *Hydrolysis of proteoglycan*

Cartilage proteoglycan was hydrolysed in sealed tubes, under N_2, with 4 N HCl (2 ml of acid per mg of material) at 100° for 8 hr [13]. The hydrolysate was then taken to dryness on a rotary evaporator and derivatized as above.

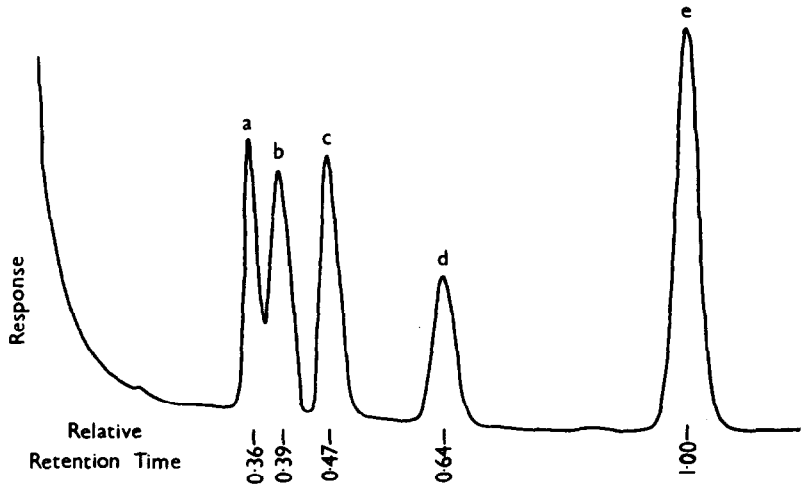

Fig. 1. A typical chromatogram of the TMS derivatives of glucosamine and galactosamine hydrochlorides. The relative retention times were calculated with phenanthrene as internal standard. (a) α-Galactosamine, (b) β-galactosamine, (c) α-glucosamine, (d) β-glucosamine, (e) phenanthrene.

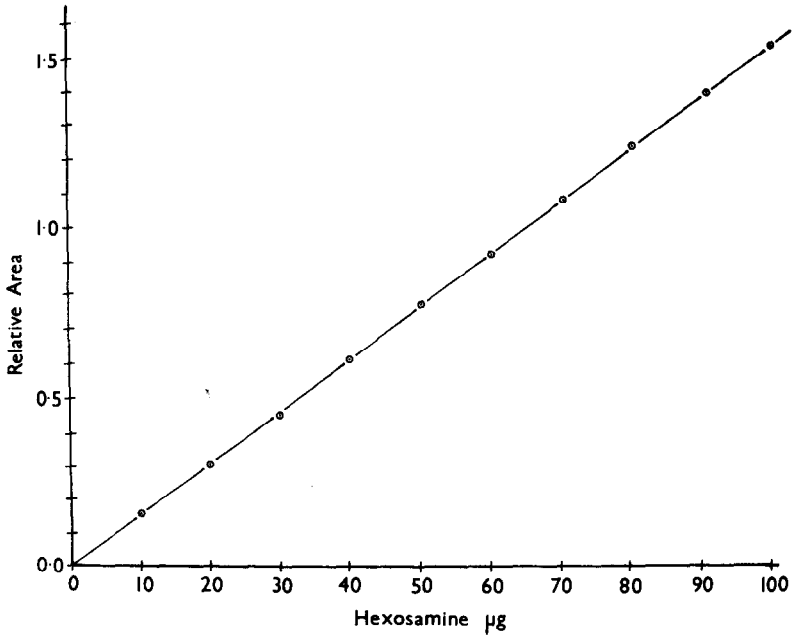

Fig. 2. Determination of hexosamines as TMS derivatives by GLC. The quantity of hexosamine shown in the figure is the total amount used for each determination. The relative area = $\frac{\text{total area of the peaks of } \alpha\text{- and } \beta\text{-anomers}}{\text{area of phenanthrene peak}}$.

Table 1
Determination of hexosamines in cartilage proteoglycan.

Proteoglycan μg dry wt.	Determined by GLC*				Determined as by Cessi and Piliego [2] *	
	Glucosamine (μg)	Galactosamine (μg)	Total (μg)	% Hexosamine	Total (μg)	% Hexosamine
100	2.6	23.2	25.8	25.8	27.7	26.7
200	5.8	47.6	53.4	26.7	51.0	25.5
300	8.9	70.6	79.5	26.5	76.2	25.4
			Average = 26.3%		Average = 25.9%	

* Average of 4 determinations.

3. Results and discussion

Various volumes of BSTFA and dimethylacetamide were investigated for the complete silylation of 100 μg of hexosamine, and it was found that complete derivatization could be obtained with 0.1 ml of a 25% solution of BSTFA in dimethylacetamide. When temperatures of reaction were examined it was observed that above 110° some degradation of the hexosamines occurred. It was therefore decided to conduct the derivatization at 100° and at this temperature the reaction reached completion after 10 min.

A typical chromatogram of the TMS derivatives of glucosamine and galactosamine is shown in fig. 1. It can be seen that peaks corresponding to the α-D- and β-D-anomers of both hexosamines are present in the chromatogram. It was observed, however, that if galactosamine was derivatized without having first been solubilized in water, only one peak, corresponding to that of the β-D-anomer, was produced. The relative retention times were calculated with phenanthrene as standard.

Samples of the hexosamines, with concentrations ranging from 10 to 100 μg, were analysed and a standard curve was made (fig. 2). The total area of the peaks of the α- and β-anomers of the hexosamines was found to be proportional to the concentration of the amino sugar and the response for each hexosamine was identical. Three analyses for each hexosamine were made at every concentration and a mean deviation of 1.3% was observed. The actual amount of each hexosamine injected into the column corresponded to 0.1–1 μg, but by altering the sensitivity of the instrument, this range may be varied. The minimum detectable amount of the TMS derivatives that can be detected in the flame ionization detector at a signal/noise level of 3:1 was found to be 7–9 × 10^{-12} moles of each hexosamine injected.

In order to test this method on biological material, samples of cartilage proteoglycan were hydrolysed, as previously described, and analysed for hexosamines by both GLC and the colorimetric method of Cessi and Piliego [2]. It can be seen from table 1 that both methods are in good agreement and that the GLC method gives consistent results over the range of material examined. The quantities of glucosamine and galactosamine determined in the bovine nasal proteoglycan are consistent with previous reports [13,14].

Under the conditions employed for hydrolysis and silylation, amino acids and small quantities of hexoses present in the proteoglycan did not cause interference. However, proteoglycan is known to contain approximately 4.5% galactose [13] and a small diffuse peak attributable to this was noticed in the chromatogram. It might therefore be necessary to remove hexoses from hydrolysates, if present in large quantities, before analysis of the amino sugars. This may readily be accomplished by passing the hydrolysate through a cation exchange resin and eluting off the hexosamines with acid.

This method provides a rapid and sensitive procedure for the simultaneous analysis of glucosamine and galactosamine in biological materials.

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