Journal of Chromatography, 94 (1974) 298–301

CHROM. 7455

Note

Rapid gas chromatographic analysis of hexosamines in body fluids*

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(Received February 19th, 1974)

D-Glucosamine and D-galactosamine are the most universally found hexosamines in nature. Specific applications of gas chromatography (GC) to the measurement of minute quantities of biological hexamines are of clinical and biomedical interest.

Since hexosamines are rather polar and poorly volatile, a variety of derivatizing methods have been used to volatilize them prior to GC injections[1]. Quantitative GC analysis of hexosamines generally requires a two-step procedure for N-acetylation and O-trimethylsilylation[2,3] or a direct trimethylsilylation of the hexosamines into tetra-O-trimethylsilyl derivatives[4] without the substitution of the amino group. The precision of the procedure could be further improved and simplified by use of more potent silylation reagents such as N,O-bis(trimethylsilyl)acetamide or its trifluoro analog, bis(trimethylsilyl)trifluoroacetamide[5].

The development of the GC-nitrogen detection system has recently been demonstrated to be a powerful and sensitive tool for the qualitative and quantitative analysis of TFA butyl amino acids[6–8] and peptide[9] and TFA derivatives of vitamins[10] and urea[11] in the biological samples. The present work was directed towards developing a rapid and specific method for the quantitative determination of hexosamines in the biological fluids.

MATERIALS AND METHODS

Solutions (10 m*M*) of D-hexosamines (National Biochemicals, Chicago, Ill., U.S.A.) were prepared in 0.1 *N* HCl. Nitrobenzene (0.5 m*M*) in hexane was used as internal standard. Rabbit seminal fluids and human urine were first hydrolyzed in 3 *N* HCl (1:1 dilution with 6 *N* HCl) at 145° for 1 h. A 40-μl aliquot of the acid hydrolysates or 5 μl of the standard hexosamines were introduced into a cone-shaped micro-vial (0.5-ml volume) for direct acylation[12] without prior cleanup. The preparation of the acyl derivatives included: (1) drying the samples at 70° under a stream of dry nitrogen, (2) adding 5 to 10 μl of internal standard, (3) adding 100 μl of dichloromethane–trifluoroacetic anhydride (3:1), (4) screwing vial with PTFE-lined cap, (5) high-speed mechanical mixing (against the inner edge of the Neoprene head) for 30 sec, (6) ultrasonic mixing for 1 min, and (7) acylation at 100° for 15 min.

* Journal Series No. 1727 of the Hawaii Agricultural Experiment Station.

A Tracor Model MT-220 gas chromatograph, four-column oven, equipped with a Coulson electrolytic conductivity detector was used in this study. The column packing and the instrumental parameters used in the GC analysis for hexosamines are outlined in Table I.

TABLE I

GAS CHROMATOGRAPHIC CONDITIONS FOR THE SEPARATION OF HEXOSAMINES

Column	0.325% (w/w) EGA* on 80–100 mesh HT Chromosorb W AW (preheated at 140° for 12 h)
Inlet temperature, °C	210
Column temperature, °C	110–190 (5°/min, programmed)
Helium flow-rate, ml/min	
Carrier	60
Pyrolyzer	10
Hydrogen flow-rate, ml/min	
Pyrolyzer	50
Pyrolyzer temperature, °C	820
Chart speed, in./min	0.5

* EGA = Ethylene glycol adipate.

Fig. 1 shows the typical GC chromatograms of the standard mixture, urine, and semen analyses. The total elution time (temperature programmed) is less than 20 min. The relative retention times were also indicated in the chromatograms. Under the experimental conditions, D-glucosamine formed only one major peak. On the other hand, α-D- and β-D-anomers of galactosamine are present as shown in the standard GC curve. It is important to note that the elution and retention of the internal standard so chosen should be free from interference of other organic N-constituents.

The data in Table II show the relative yield of five independent trials for glucosamine, galactosamine and total hexosamine in terms of peak height ratio, component peak(s) of interest *vs.* internal standard (not being derivatized). These data reflect the basis for reproducibility and quantitation when the internal standard method is used. The average peak height ratio of each or total hexosamine showed a standard devi-

TABLE II

DETERMINATION OF HEXOSAMINE RELATIVE YIELD BY PEAK HEIGHT RATIO OF HEXOSAMINE *vs.* INTERNAL STANDARD*

Trial	*Glucosamine***	*Galactosamine***	*Total hexosamine*
1	3.19	3.33	6.52
2	3.17	3.34	6.51
3	3.15	3.37	6.52
4	3.20	3.33	6.53
5	3.18	3.33	6.51
Average	3.18 ± 0.02	3.34 ± 0.02	6.52 ± 0.00

* Nitrobenzene = 1 (10 μl of 0.5 mM per vial).

** 5 μl of 10 mM in 0.1 N HCl per vial.

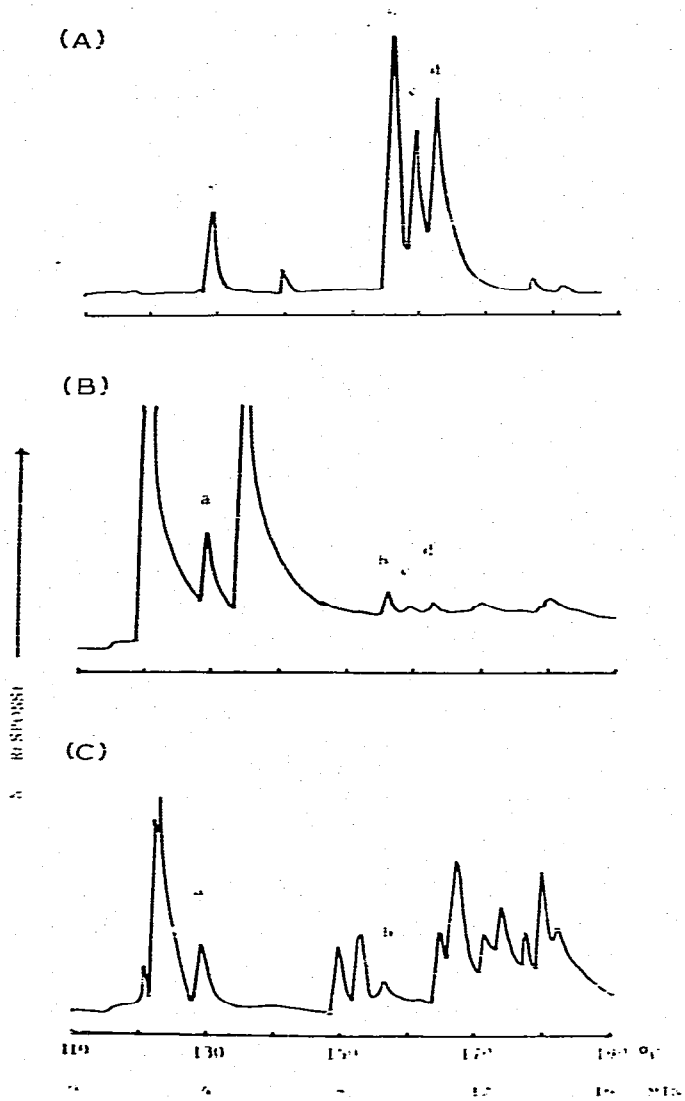

Fig. 1. Gas chromatographic curves showing the elution, the relative retention, and the hexosamines to internal standard yield ratio in (A) standard mixture, (B) acid hydrolyzed urine (1 μl), and (C) acid hydrolyzed rabbit semen (1 μl). a = Nitrobenzene (internal standard); b = glucosamine; c = α-D-galactosamine; d = β-D-galactosamine.

ation range between zero to $\pm$0.02. This precision is satisfactory for its applications.

A recovery study was carried out by adding increasing amounts of hexosamine mixture (1–5 μl of 10 mM) to the urine acid hydrolysates samples (40 μl) and by measuring the increases in response due to these additions as compared to the response of samples with zero addition. Recovery values for glucosamine, galactosamine and total hexosamine ranged from 92 to 99%.

If a constant amount of internal standard is used in each sample vial, a simple quantitative calculation of hexosamine in the biological sample can be obtained by the following formula:

$$\text{Hexosamine(s), mg per 100 ml of sample} = \frac{\text{peak ratio in sample}}{\text{peak ratio in standard}} \times \frac{\text{wt. of std. in calibration mix}}{\mu\text{l of sample}} \times 100$$

The procedure of internal standard method (peak ratio in test sample) is also helpful in determining the optimal conditions (time, temperature, and acid strength) of acid hydrolysis which become necessary for the analysis of hexosamines in mucopolysaccharides.

ACKNOWLEDGEMENT

This work was supported in part by a research grant from the Research Council, University of Hawaii.

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