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Note

Improved method for the determination of hexosamines using the Beckman 121-M amino acid analyzer

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Until recently, the usual method for hexosamine determination has been by the colorimetric Elson–Morgan reaction, or a modification thereof[1]. However, this procedure is subject to interference by amino acids, other sugars, and degradation products produced during acid hydrolysis[2]. The Elson–Morgan reaction, while quantitative for total hexosamine content, cannot be used to distinguish between individual amino sugars without cumbersome modifications to the procedure[3,4].

Paper chromatography and thin-layer chromatography (TLC) have been used to separate and identify glucosamine and galactosamine. However, quantitation by these methods remains difficult[5,6].

Separation of hexosamines has been accomplished by ion-exchange chromatography[7,8] and when combined with a modified Elson–Morgan reaction has been utilized for the determination of distinct hexosamines in the column effluent[8]. The automated amino acid analyzer has also been used for individual hexosamine determinations[9–11]. However, these methods are time consuming and sensitive to only microgram quantities of each amino sugar[10].

Methods employing pre-column derivation of hexosamines have been reported for gas chromatography[12] and for high-performance liquid chromatography[13]. While these methods demonstrate increased sensitivity, they require laborious sample pre-treatment and derivation.

The method presented is an improved analytical procedure for the separation and quantitation of the hexosamines, glucosamine, galactosamine, and mannosamine using the amino acid analyzer. The analysis is free from interfering compounds. The procedure is rapid and at least as sensitive as previously presented pre-column derivation methods.

MATERIALS AND METHODS

The standard hexosamines, D-glucosamine–HCl, D-galactosamine–HCl, and D-mannosamine–HCl, were obtained from Sigma (St. Louis, MO, U.S.A.). The glycoprotein, fetuin, isolated from calf-serum, was obtained from Calbiochem (La Jolla, CA, U.S.A.). Human hard keratin fibers (hair) were taken from a single donor. The cation-exchange resin, AG 50W, was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.).

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Hexosamine separation and determination was performed on a dual column Beckman 121-M amino acid analyzer. Each column, 15 cm × 2.8 mm I.D., was packed with Beckman W-2 resin. Column temperature was maintained at 65.0°C. Each analysis employed a 50 μl sample volume. A slightly modified sodium (0.07 *M* Na^+) borate–citrate buffer, pH 7.24 (ref. 9), was used to elute the hexosamines from the cation-exchange column. The flow-rate of the buffer was 8.8 ml/h, with a column back pressure of 1.0 MPa. Hexosamines were derivatized by a Beckman ninhydrin reaction system and the ninhydrin reactive compounds were subsequently detected for absorbance measurements at 570 nm with a 0.2 O.D. full scale deflection. Peak area quantitation was performed by an in-line Beckman integrator (System AA). A hexosamine standard mixture containing 100 nmoles/ml each of mannosamine, glucosamine, and galactosamine was used to calibrate each amino sugar derivative's absorbance response.

Two hexosamine determinations can be made within 90 min using the automated dual column amino acid analyzer. All hexosamines eluted within 50 min. As hexosamines were eluted from one column, the other column was regenerated for 19 min with 0.2 *M* sodium hydroxide solution), then equilibrated for 28 min with the borate–citrate buffer to prepare the column for the next analysis. The program-controlled analyses can be repeated continuously. This automated dual column system facilitated the rapid routine analysis of glucosamine and galactosamine.

In order to test this method for accuracy and possible interference from amino acids and/or peptides, two model biological materials, fetuin glycoprotein and hard keratin fibers (HKF), were analyzed for their hexosamine content. Keratin was used as a particularly hydrolysis-resistant material, low in hexosamine content, from which it is difficult to extract the amino sugars.

Acid hydrolysis was used to release the hexosamines from the sample matrix. After hydrolysis with 6 *M* hydrochloric acid at 100°C, each sample was allowed to cool to room temperature, and 100 nmoles of mannosamine was added as an internal standard. Hydrochloric acid was removed in a rotary evaporator under reduced pressure at 50°C. The hydrolyzate was dissolved in water and applied to a 3 × 1 cm AG 50W-X8 (200–400 mesh) H^+ column. The column was washed with 5 ml of water and the hexosamines then eluted by the addition of 5 ml of 2 *M* hydrochloric acid. This preparative measure rid the sample of most interfering acidic and neutral compounds. The acid effluent was dried down and redissolved in 1.0 ml of distilled water. The sample was subsequently analyzed for hexosamines on the amino acid analyzer as described previously.

The optimum conditions under hydrolysis for maximal release of hexosamines from fetuin and HKF were then established by hydrolyzing each material for varying amounts of time with 3.5 ml of 6 *M* double distilled hydrochloric acid *in vacuo* at 100°C.

RESULTS AND DISCUSSION

A typical chromatogram showing the separation of a standard mixture of mannosamine, glucosamine, and galactosamine is presented in Fig. 1. The retention times of these sugars were 26.0, 34.5, and 44.0 min, respectively. All peaks were baseline resolved, and each hexosamine derivative gave comparable absorbance re-

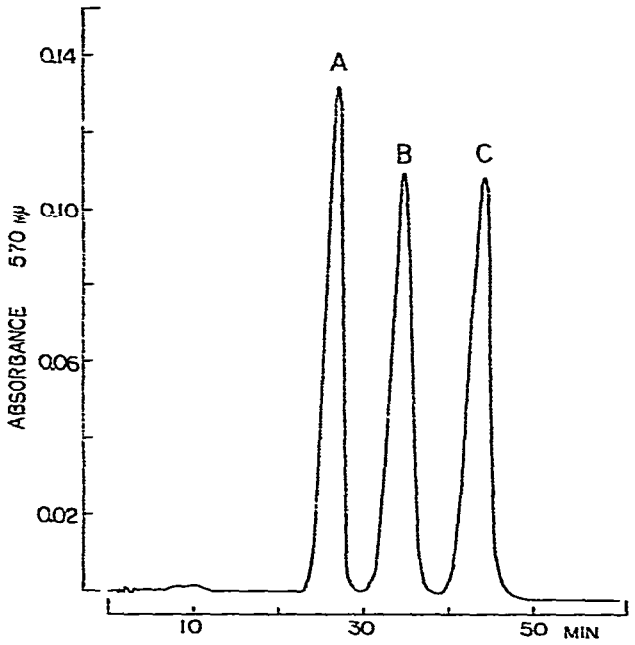

Fig. 1. Chromatograph of a standard mixture of 5 nmoles each of (A) mannosamine, (B) glucosamine, and (C) galactosamine. A sodium (0.07 *M* Na^+) borate–citrate buffer is used to elute the hexosamines at a flow-rate of 8.8 ml/h. Column temperature is 65.0°C. Detection is at 570 nm.

sponse. This response was linear within the domain of 0.25 to 5.0 nmoles of each sugar applied to the column. The amino sugars can be analyzed even when their relative molar ratios are as great as 20 to 1.

The release of hexosamines from fetuin and HKF by acid hydrolysis was maximal at 4 and 16 h, respectively.

Fetuin gave a linear release of hexosamines from 0.1 to 1.0 mg of the glycoprotein hydrolyzed for 4 h (Fig. 2). In contrast, HKF gave a linear release of amino sugars from 5 to 40 mg of sample hydrolyzed for 16 h (Fig. 3).

Chromatograms of fetuin and HKF hydrolyzates are shown in Figs. 4 and 5. Acidic and neutral amino acids eluted well before mannosamine. Under these column conditions, basic amino acids were not eluted from the column with the borate–citrate buffer. Interfering peptide peaks did not occur under these conditions when hydrolyzate samples were pre-treated by passing the sample through AG 50W resin. Chromatograms of HKF had well resolved peaks and showed no interfering material even when 40 mg hydrolyzate samples were analyzed (chromatogram not shown).

The amounts of each individual hexosamine found in fetuin and HKF are presented in Table I. All values were calculated from the slopes of the curves in Figs. 2 and 3 by linear regression analysis. The hexosamine values for calf-serum fetuin compare closely with those reported by Spiro[14]. Hydrolysis conditions for the complete release of each hexosamine from the sample matrix must be tailored to the individual glycoprotein being analyzed. The release of glucosamine from fetuin can be raised from 86% to 100% by altering the acid concentration, the temperature, and

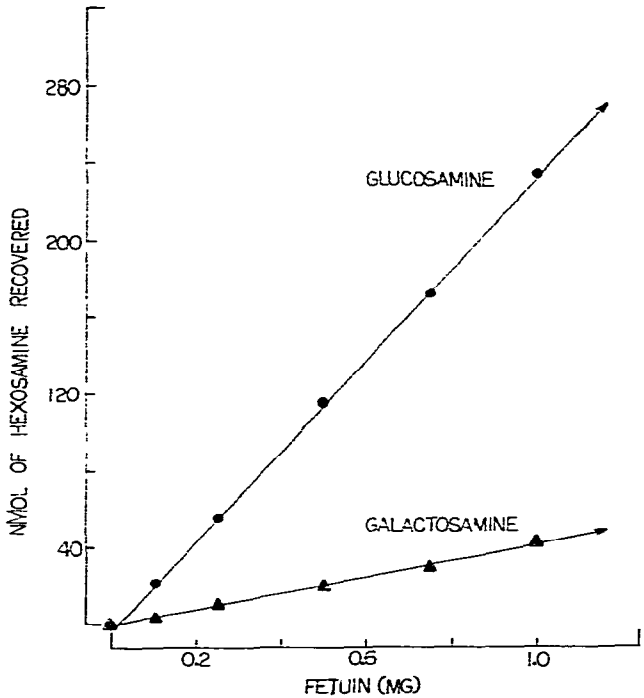

Fig. 2. Proportional recovery of glucosamine and galactosamine from varying amounts of fetuin hydrolyzed. Hydrolysis is with 6 *M* hydrochloric acid *in vacuo* at 100°C for 4 h. Each data point is the mean of three determinations. Actual sample size analyzed was from 5 to 50 μg of fetuin in a 50-μl sample volume.

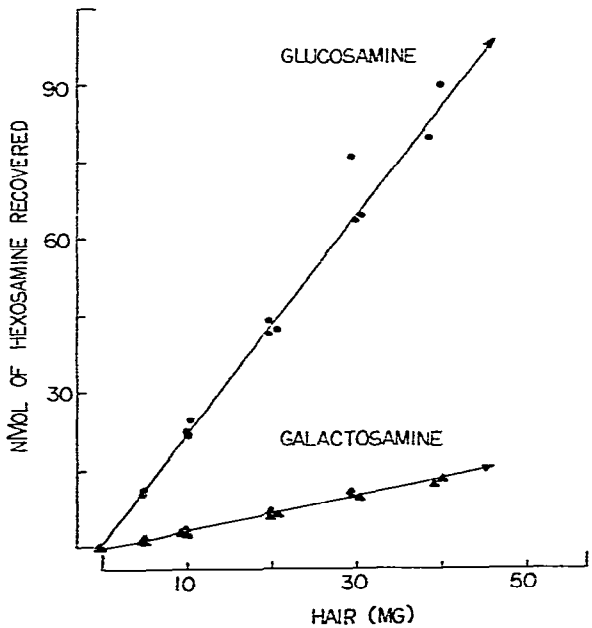

Fig. 3. Proportional recovery of glucosamine and galactosamine from varying amounts of hard keratin fibers hydrolyzed. Samples were hydrolyzed with 6 *M* hydrochloric acid *in vacuo* at 100°C for 16 h.

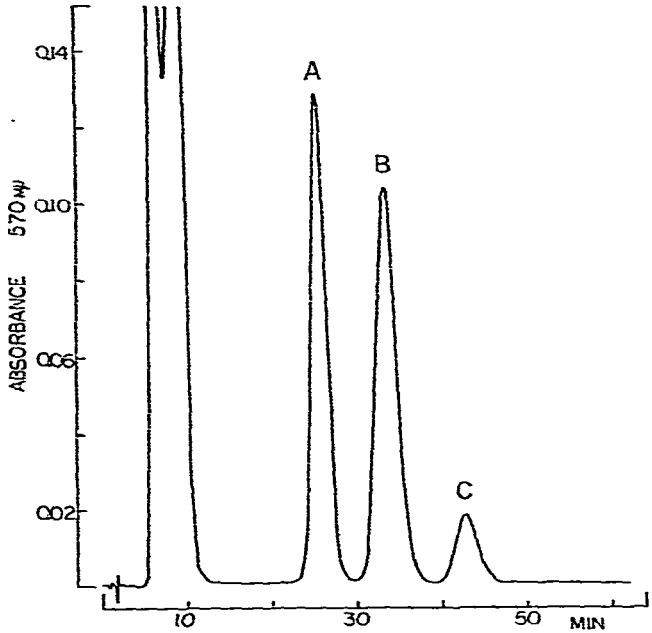

Fig. 4. Chromatograph of a fetuin hydrolyzate. Order of elution: (A) internal standard (mannosamine), (B) glucosamine, and (C) galactosamine. Actual sample size analyzed is 25 μg of fetuin in a 50-μl injection volume. Chromatographic conditions are as stated in Fig. 1.

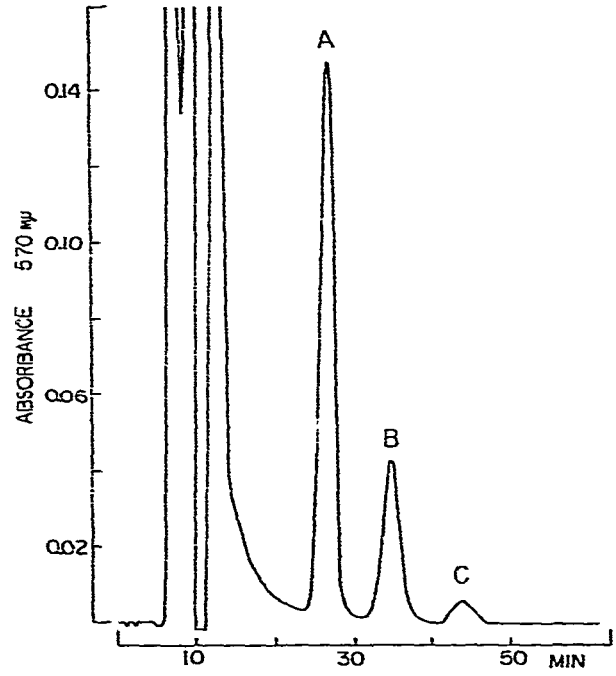

Fig. 5. Chromatograph of a hard keratin hydrolyzate. Order of elution: (A) internal standard (mannosamine), (B) glucosamine, and (C) galactosamine. Actual sample size analyzed is 1.0 mg of HKF in a 50-μl sample volume. Chromatographic conditions are as stated in Fig. 1.

TABLE I

CONCENTRATION OF HEXOSAMINES IN SAMPLES

Values are reported as % weight of hexosamines as free bases. They were calculated from the slopes of the curves in Figs. 2 and 3 by linear regression analysis.

Sample	*Found*		*Reported**	
	GlcN (%)	*GalN (%)*	*GlcN (%)*	*GalN (%)*
Fetuin (isolated from calf-serum)	4.2	0.7	4.9	0.6
Hard keratin fibers (human)	0.039	0.006	–	–

* Ref. 14.

the time needed for hydrolysis. The hexosamine values given in the table for HKF are the first reported values to our knowledge.

The reproducibility of the procedure was demonstrated by the precision of the hexosamine analyses of fetuin of varying sample concentration (see Figs. 2 and 3). Linear regression analysis of the data gave correlation coefficients (r^2) of 0.9997 for glucosamine and 0.9992 for galactosamine. The coefficients of variation (standard deviation of hexosamine concentration/mean hexosamine concentration) for the amounts of hexosamines in fetuin were 2.9 and 4.3% for glucosamine and galactosamine, respectively. These latter values correlate favorably with those reported for hexosamine analysis by gas chromatography[12].

Preliminary chromatography of samples revealed the occurrence of hexosamine peak splitting. This proved to be problematic for accurate quantitation of the sugars. Extensive analysis showed the cause to be with the AG 50W preparative resin. Unless the resin was sufficiently washed before use with three volumes each of 2 *M* hydrochloric acid and distilled water, the doublets would continue to occur. This was possibly due to the inefficient removal of sodium ions, remaining from the regeneration of the resin with sodium hydroxide. The interaction of free cations, such as sodium, with the *cis*-hydroxyl groups of the hexosamines has been postulated as a cause of multiple peaks[13,15]. In fact, it has been proposed that the peak doublets are the hexosamines' α and β anomers[11]. Peak splitting was not observed for standard mixtures of hexosamines directly chromatographed on the amino acid analyzer. However, peak splitting of standard mixtures of hexosamines did occur if the sample was treated with unwashed preparative resin. Sodium cation association with the sugars is most likely inhibited if the hexosamines first form a hexosamine–borate complex, made possible by the borate–citrate buffer used to elute the sugars.

The procedure presented has been specifically designed for the rapid separation and sensitive determination of biologically common hexosamines within a complex mixture of compounds. It is an improvement over past methods for hexosamine determination using the amino acid analyzer. Hexosamines can be analyzed in the presence of excessive amounts of amino acids and peptides, such as is the case with hard keratin hydrolyzates. In addition, sample sizes as small as 5 μg can be analyzed with a sensitivity to 0.25 nmoles of both glucosamine and galactosamine.

The automated dual-column amino acid analyzer was found to be ideal for routine hexosamine analysis. The post-column derivation system permits collection

of amino sugars before reaction with the ninhydrin reagent, and avoids the instability of pre-column hexosamine derivatives.

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