Improved Continuous-Flow Method for Determination of Total Serum Hexosamines


We describe an automated determination of serum hexosamine by the Elson–Morgan reaction, together with reagent modifications that minimize interference from amino acids and sugars present in acid hydrolysates of sera. We used a novel 15-min autoclave procedure for the acid hydrolysis of sera before analysis to facilitate the determination as compared to the 4-h hydrolysis used in the conventional manual method. Results correlated well ($r = 0.906$) with those obtained by the corresponding manual method.

The potential prognostic or diagnostic importance of disease-induced variations in carbohydrate components (hexosamine, neutral hexoses, and L-fucose) of serum glycoproteins (1, 2) has generated a need to facilitate such analyses.

Balazs et al. (3) proposed an automated method for hexosamine determination. We found their procedure to be quite suitable for pure aqueous hexosamine solutions but unsuitable for the direct analysis of serum because of interferences from amino acids and sugars normally present in acid hydrolysates of biological material. Similar interference has been reported by others (4, 5). Their method and the one described here is based on the Elson–Morgan reaction (6). We have modified the concentrations of acetylacetone, hydrochloric acid, ethanol, and $p$-dimethylaminobenzaldehyde as used by Balazs et al. (3) to minimize interference from these sources. We also used sodium carbonate as originally proposed by Elson and Morgan (6) rather than trisodium phosphate (3) to create an alkaline medium for the acetylation step of the reaction. Finally, use of a novel 15-min autoclave procedure (7) shortens hydrolysis time to 15 min instead of 4 h (8).

**Materials and Methods**

**Reagents and Standards**

The following reagents were used in developing the proposed procedure.

- **$\text{Na}_2\text{CO}_3$ solution, 0.25 mol/liter.**
- **Acetylacetone** (Sigma Chemical Co., St. Louis, Mo. 63178), 20 ml/liter in 0.25 mol/liter $\text{Na}_2\text{CO}_3$; final pH, 9.5. Solution should be colorless. Prepare daily.
- **Ehrlich reagent:** Dissolve 0.4 g of $p$-dimethylaminobenzaldehyde (Sigma) in a mixture of 195 ml of ethanol and 45 ml of concentrated HCl. Prepare daily.

Glucosamine hydrochloride standard solutions (Sigma) prepared to span the range 25–200 mg/liter (in terms of the free amino sugar).

All other reagents were reagent grade. The AutoAnalyzer system (Technicon Instruments Corp., Tarrytown, N. Y. 10591), consisting of a Sampler II, Proportioning Pump, Heating Bath (95 °C), Colorimeter, and Recorder, was used in the procedure. Human serum samples were obtained from the Naval Hospital, National Naval Medical Center, Bethesda, Md., as part of a continuing study for differentiating malignant neoplasia from nonmalignant diseases (1).

**Procedure**

Serum samples, 0.2 ml, were hydrolyzed with 2 ml of 3 mol/liter HCl for 15 min in an autoclave operated at 103.4 kPa (15 lb/in.$^2$) and 122 °C (7). After cooling to room temperature, the hydrolysates were neutralized with 3 mol/liter NaOH [barely alkaline to litmus (8)]. Any precipitate—some occasionally occurs during the hydrolysis and neutralization steps—must be removed either by centrifugation or filtration$^1$ before the sample is analyzed.

Figure 1 is a diagram of the flow-manifold for the procedure. Good resolution of recorder peaks was obtained with the sampling rate shown. The final color is measured at 520 nm in a 15-mm tubular flowcell. Much carbon dioxide (6) is developed during the reaction, but this does not interfere with the procedure. A minor rhythmic fluctuation of the baseline is attributed, in part, to variations in heating-bath temperature during the 95 °C heating step of the procedure.

**Results and Discussion**

**Analytical Variables**

The interference produced by mixtures of amino acids and neutral hexoses found in acid hydrolysates of biological material can reportedly (5) be eliminated in the case of some biological samples by performing the reaction at pH 9.5 and increasing the concentration of acetylacetone in 0.25 mol/liter sodium carbonate from about 20 ml/liter, as employed by Elson and Morgan (6), to about 60 ml/liter. We did not find this to be true for serum analyses. Although we did not specifically study the effect of pH on the reaction, the apparent optimal pH for glucosamine, as reported by others [cited by Balazs et al. (3)], is between 9.5 and 9.7 and for sugar/amine mixtures between 10.8 and 11.2 (5); therefore we used a pH 9.5.

$^1$ We find that filtration through disposable 0.45 μm filters ("Swinnex-13"; Millipore Corp., Bedford, Mass. 01370) is the most effective way to remove precipitate.
Fig. 1. Flow manifold for the automated determination of serum hexosamines
Designation of tube sizes and connectors are those of the supplier (Technicon Corp.)

Fig. 2. Effect of various concentrations of acetylacetone on the absorbance of chromogens produced by glucosamine and a synthetic mixture of lysine and hexose
Glucosamine concentrations: curve A, 100 mg/liter; curve B, 50 mg/liter. The synthetic mixture is shown as curve C; its composition is described in the text.

of 9.5, rather than pH 10.2 as used by Balazs et al. (3), to create the alkaline medium for the acetylation step. Our results (Figure 2) indicate that the 20 ml/liter concentration of acetylacetone suggested by Elson and Morgan (6) gives the optimum color yield for glucosamine (curves A and B); increasing the acetylacetone concentration beyond 20 ml/liter decreases the color intensity of glucosamine but increases the color intensity of a synthetic mixture (curve C) of lysine (8.71 g/liter) and equimolar galactose/mannose (1.21 g/liter). The composition of this synthetic mixture was predicated on the following assumptions: (a) human serum proteins contain no more than 12% lysine (9), which is the major interfering amino acid (5, 10) present in serum proteins; (b) the total protein content of human serum is about 70 g/liter; and (c) the mean concentration of serum protein-bound neutral hexoses is 1.20 g/liter (8). This synthetic mixture was used undiluted.

The composition of Ehrlich's reagent was varied by trial and error to minimize the absorbance produced in the Elson-Morgan reaction with the synthetic mixture described above. Reagents prepared as described above effectively decreased the interference to what we consider acceptable levels. The actual absorbance of standard glucosamine solutions with (A) and without (B) the added synthetic mixture is shown in Table 1. This interference is further decreased about 20-fold in the analytical procedure because sera are diluted during the hydrolysis and neutralization steps.

Table 1. Comparison of Absorbance of Glucosamine Standard Solutions with (A) and without (B) Addition of a Synthetic Mixture of Lysine and Neutral Hexoses

<table>
<thead>
<tr>
<th>Glucosamine, mg/liter</th>
<th>Absorbance (520 nm)</th>
<th>A</th>
<th>B</th>
<th>A - B</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.165</td>
<td>0.159</td>
<td>+0.006</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.273</td>
<td>0.271</td>
<td>+0.002</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>0.371</td>
<td>0.361</td>
<td>+0.010</td>
<td></td>
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<tr>
<td>200</td>
<td>0.439</td>
<td>0.435</td>
<td>+0.004</td>
<td></td>
</tr>
</tbody>
</table>

* Lysine (8.71 g/liter) and equimolar galactose/mannose (1.21 g/liter).

Fig. 3. Calibration curve obtained for glucosamine standards for measurements at steady state.

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in the procedure is about 80% of that measured at steady state. Color intensity produced by galactosamine is about 86% of that exhibited by glucosamine. Glucosamine was used as the standard because it comprises 86–97% of total serum hexosamines (11).

Automated and Manual Procedures Compared

Values ranging from 0.60 to 1.60 g/liter, obtained with 30 human serum samples assayed by the proposed automated method and by the manual method (8), had a correlation coefficient of 0.906; the regression equation was y = 0.850x + 17.0, (y = manual, x = automated). The slope and intercept did not differ significantly from 1.0 and 0, respectively. Results of replicate analyses of a commercial control serum (“Monitrol XLT34”; Dade Division, American Hospital Supply Corp., Miami, Fla. 33125) by the automated (0.93 ± 0.06 g/liter) and manual (0.88 ± 0.09 g/liter) procedures indicate that no practical difference exists when mean values obtained by both methods are compared.

References


Unsuitability of Porcelain Crucibles for Dry Ashing of Biological Tissues for Calcium Analysis

Martha Meyer and Ralph A. Meyer, Jr.

At temperatures required for complete release of calcium from beef liver by dry ashing, porcelain crucibles release significant amounts of calcium into the ash, which leads to erroneously high calcium values in the samples. Heated, empty porcelain crucibles do not show released calcium. Both fused silica and platinum crucibles fail to show any significant interaction with the sample. Samples dry-ashed in fused silica crucibles were compared to samples subjected to the hot trichloroacetic acid–acetic acid extraction method, which gave results not significantly different if the tissue was first minced. However, calcium was incompletely extracted by the hot acid from whole segments of liver weighing about 0.2 g.

Assays of cellular calcium usually require removal of the calcium from the organic matrix, commonly by dry ashing in crucibles at temperatures exceeding 500 °C. Crucibles are available in a variety of materials, but we know of no studies on the validity of use of various crucible materials in calcium assays.

Porcelain crucibles are inexpensive and their use has been reported in a "house" publication (1). Porcelain crucibles are also recommended by the Perkin-Elmer Corp. in a widely used manual of standard procedures for calcium assay by atomic absorption (2, 3). Fused silica crucibles (4) and platinum crucibles (5) are also used.

We encountered problems with porcelain crucibles that invalidate their use in calcium assays. In a search for a suitable alternative, porcelain crucibles were compared to platinum and fused-silica crucibles. The hot trichloroacetic acid (TCA)–acetic acid extraction method (6, 7) was also compared to results obtained by dry ashing.

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