Determination of α-Sorbitol in Human Erythrocytes by an Improved Enzymatic Method with Fluorometric Detection

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In this enzymatic method to analyze erythrocytes for α-sorbitol, the erythrocytes were separated from plasma by centrifugation, washed with isotonic saline (9 g/L NaCl), then diluted threefold with more saline. We lysed 1.5 mL of the diluted erythrocytes with chloroform and precipitated the protein with 58 g/L HClO₄ solution. The resulting supernatants were mixed with buffer, NAD⁺, and sorbitol dehydrogenase (EC 1.1.1.14). For standards, we added sorbitol to the erythrocytes before lysing. After 25 min of incubation at 37 °C, the fluorescence responses were recorded. Results for sorbitol were corrected for sample blank and enzyme fluorescence, and were then normalized for hemoglobin content. Response was linear for a sorbitol concentration range of 1 to 30 μmol/L. Reproducibility was good, with an average CV of 2.5% at 10 μmol/L. Results for healthy individuals and diabetic patients are presented.

Additional Keyphrases: diabetes · hemoglobin · glucose

Cataract and other complications resulting from diabetes mellitus are believed to be caused by the accumulation of α-sorbitol. The polyol pathway and the role of aldose reductase in the in vivo synthesis of sorbitol from glucose have been described elsewhere (1–3). Concentrations of sorbitol have been reported in the lens cells and erythrocytes of diabetic patients and experimentally diabetic rats (3–6). As sorbitol accumulates in the lens cells, osmotic balance is disrupted, leading to changes in water content, metabolism, and cell structure (7). Eventual lens opacity may be due to protein aggregation (3) or rupture of lens fibers (7, 8).

Because published analytical methods for quantifying sorbitol in erythrocytes (9–13) were not suitable for assaying...
ing the large number of samples involved in clinical studies, we modified the enzymatic assay of Malone et al. (9). In their assay, separated erythrocytes were washed three times with saline and lysed by freezing and thawing three times. To speed the sample preparation, we used a single saline wash and lysed the cells with chloroform. The pH 9.4 glycine buffer they (9) used was removed and the reaction run at neutral pH. This decreased background and improved the assay specificity. Sorbitol response was measured after having plateaued, so that precise timing for measurements after incubation was unnecessary.

Materials and Methods

Materials

Instruments. We used a Model 650-10S fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, CT) with a Model D5517-5 Omniscan® strip-chart recorder (Houston Instruments, Austin, TX) for all fluorescence measurements. Excitation and emission wavelengths were set at 343 and 455 nm, respectively. Slit width was 10 nm for both excitation and emission. Samples were read in 1 × 1 cm disposable cuvettes (Evergreen Scientific, Los Angeles, CA). For centrifugation we used a Model TJ-6 refrigerated centrifuge (Beckman Instruments, Fullerton, CA) at 1100 × g and 4°C.

Reagents. D-Sorbitol, sorbitol dehydrogenase (EC 1.1.1.14; l-iditol dehydrogenase), and NAD⁺ (β-nicotinamide adenine dinucleotide, Grade V, from yeast) were obtained from Sigma Chemical Co., St. Louis, MO 63178. Polyols and monosaccharides used in the interference studies were purchased from The Foxboro Co., North Haven, CT 06473. All other chemicals used to prepare the reagents were purchased from J. T. Baker Chemical Co., Phillipsburg, NJ 08865. Purified water was prepared in-house, with a Barnstead "Nanopure I" water system (Sybron/Barnstead, Boston, MA 02132).

Reagent Preparation

Chloroform. Before use, wash the chloroform with purified water by shaking equal volumes of chloroform and water in a separatory funnel, discarding the water each time. Store the chloroform in glass at 4°C.

Stock phosphate solution. Dissolve 0.1 mol of tribasic sodium phosphate dodecahydrate in 200 mL of purified water.

Blank. Dilute 0.9 g of sodium chloride and 20 mL of the stock phosphate solution to 100.0 mL with purified water.

Buffer. Adjust the stock phosphate solution to a pH of 11.0 with hydrochloric acid (6 mol/L).

NAD⁺ solution. Dissolve 1 g of magnesium acetate tetrahydrate and 160 mg of NAD⁺ in 95 mL of purified water. Adjust the pH to 5.5 ± 0.3 with hydrochloric acid (6 mol/L) then dilute the solution to 100.0 mL with purified water.

Enzyme. Dilute the sorbitol dehydrogenase, as purchased, with cold (4°C) water to an activity of 5.75 to 6.75 kU/L. A typical dilution would be 50 mL per 10-mg vial.

Standards. The stock standard, prepared by dissolving sorbitol in cold water to give a concentration of 10 mmol/L, was further diluted to 300 μmol/L with cold water to prepare the working standard.

Sample Preparation

Venous blood, collected in glass tubes containing 0.07 mL of 150 g/L EDTA(K3) solution, was immediately cooled in an ice-water bath, transferred to polypropylene centrifuge tubes, and centrifuged for 15 min. After discarding the plasma anduffy coat, we added isotonic saline equal to twice the volume of the erythrocytes and gently inverted the tube to wash the cells. After centrifuging the specimen again for 15 min, we discarded the saline wash and then either assayed the samples immediately or stored them at −80°C, at which temperature they are stable for at least 16 months. Before assaying stored samples, we let them sit at room temperature until thawing began, then placed them in an ice-water bath. This approach lessened thermal shock and avoided cracking the tubes. The washed erythrocytes were then diluted with two volumes of isotonic saline and mixed by inversion several times before assay.

Procedure

Samples. For each specimen, pipet 1.5 mL of diluted erythrocytes into a 15-mL polypropylene screw-capped centrifuge tube. Add 2 mL of chloroform to the blood cells, cap, and shake the tubes vigorously for 1 min to lyse the cells. Before removing the cap, centrifuge the tubes for 1 min. Add 2.0 mL of 58 g/L perchloric acid reagent and shake for 30 s to precipitate the protein. Separate the chloroform, protein, and aqueous supernate by centrifuging for 5 min.

Transfer 1.0 mL of the supernate to a second tube and neutralize the acid (pH = 6.9 ± 0.2) with 1.0 mL of buffer. Vortex-mix for 10 s, then add 1.0 mL each of the NAD⁺ and enzyme reagents. For the sample blank, treat a second 1.0-mL aliquot of the supernate identically, but substitute 1.0 mL of water for the enzyme reagent.

Cap the tubes and mix the contents by inverting gently five times. Incubate the samples in a 37°C water bath for about 25 min, then cool at room temperature for 15 min. Measure the fluorescence produced at room temperature within 1 h, after having filtered the samples through serum filters (cat no. P5190-6; American Scientific Products, McGaw Park, IL) to eliminate any suspended particles. Set the maximum instrument response using one of the standards.

Standards. Add 50 μL (15 mmol) of the 300 μmol/L working standard to a 1.5-mL aliquot of diluted erythrocytes and assay. Standards must be prepared for each specimen, to minimize matrix effects.

Enzyme and reagent blanks. Substitute 1.5 mL of the blank solution for the dilute erythrocytes in the assay procedure. An enzyme blank is prepared by adding enzyme to the supernate; a reagent blank is prepared by using water in place of the enzyme.

Quantification. Using recorder-pen deflection (peak height; see Figure 1) as a measure of fluorescence, calculate the enzyme response, Rₜ; the net standard response, Rₛ; and the net sample response, Rₜ Rₛ. Rₜ Rₛ is the difference between the responses of the enzyme blank and reagent blank. Rₛ is calculated by subtracting the sample response from the standard response. (In the case of duplicate analysis, Rₛ is calculated by subtracting averaged sample responses from the averaged standard responses.) To calculate Rₛ, subtract each sample-blank response from its corresponding sample response. Calculate the sorbitol concentration as C × (Rₜ − Rₛ/Rₛ), where C is the concentration of the standard (about 10 μmol/L).

Hemoglobin. We determined the concentrations of hemoglobin in aliquots of diluted erythrocytes spectrophotometrically (14, 15), then compared the ratio of sorbitol concentration with hemoglobin concentration in the specimens. Be-
cause the concentrations of both sorbitol and hemoglobin are expressed as quantity per volume of diluted erythrocytes, no corrections for dilution were needed.

Results and Discussion

Accuracy. Linearity studies were done by adding increasing volumes of working standard to 1.5-mL aliquots of diluted erythrocytes. These standards were then assayed along with an un supplemented sample of diluted erythrocytes. Blanks were not prepared because we were not measuring the response of the endogenous sorbitol. We prepared four plots of the added sorbitol concentration vs the net standard response; all four curves were linear (r² > 0.998) and passed near the origin (Table 1). The different slopes reflect the fact that different blood specimens were used to prepare each curve. The effect of matrix on the enzymatic reaction necessitates that a standard be prepared for each specimen to ensure accurate results.

Precision. Replicate standards were prepared and assayed. Three analysts prepared on different days six sets of 10 standards, at concentrations of 2, 6, and 10 μmol/L. Results of the analyses are presented in Table 2.

In early validation testing of this procedure, we observed a problem in day-to-day reproducibility. Washing the chlороform with water before use eliminated this.

Interference. We prepared 10 μmol/L standards of the following polyols and sugars—xyitol, d-mannitol, dulcitol, erythritol, d-arabitol, myo-inositol, L-alabitol, adonitol, sucrose, d-fucose, d-ribose, D-fructose, L-sorbose, d-xylene, a-D-galactose, d-mannose, d-arabinose, L-arabinose, a-D-lyxose, a-D-glucose, D-D-glucose—using a common pool of diluted erythrocytes, then assayed these with a sorbitol standard as a control. We also assayed a sample of the diluted erythrocytes and calculated the net response of each standard. No significant interference was observed. We used a neutral pH, to minimize interference from these polyols and sugars. A glycine buffer at pH 9.4 produced responses from xylitol and adonitol that exceeded the sorbitol response. Lowering the pH to 6.9 reduced the response of all the polyols and sugars tested, but affected sorbitol least. Interference from adonitol was eliminated and that from xylitol was diminished.

Detection limit. We estimated the limit of detection—three times the between-peak noise—as being 0.3 μmol/L (0.05 mg/L). In a typical scan a 10 μmol/L standard produced an average net standard response of 100 mm, with peak-to-peak noise being at most 1 mm.

Timing. In determining the optimum incubation time for the samples, we found that the net response of standards reached a plateau within 20 to 25 min. Increasing the incubation time from 20 to 25 min increased the net response by 7%, whereas increasing the incubation from 25 to 60 min changed the net response by only 5%.

We also investigated the time needed to return incubated samples to room temperature. As determined by direct temperature measurement, samples at 37 °C cooled to room temperature after 15 min. Extending the cooling to 75 min produced no difference in net response. This leeway in equilibration time ensured that response did not change significantly between the first and last measurement of response and eliminated the need for precise timing during equilibration and measurement.

Stability. The stability of sorbitol in washed erythrocytes at −80 °C was investigated by aliquoting washed erythrocytes from a diabetic patient into several centrifuge tubes and storing at −80 °C. We analyzed these samples for sorbitol in duplicate at 0, 1, 2, 3, 5, and 16 months afterwards. The measured sorbitol concentration varied by only 4.5% (CV), indicating that sorbitol remains stable in the washed erythrocytes under these conditions at least 16 months.

Method application. To establish baseline values for clinical studies, we analyzed blood from 23 normal fasting subjects and 28 diabetic subjects for sorbitol and hemoglobin. The mean sorbitol/hemoglobin ratio was 14.4 (SD 3.8) nmol/g and 27.4 (SD 17.1) nmol/g, respectively. Analyses of additional blood samples for sorbitol, hemoglobin, and plasma glucose (Figure 2) showed a statistical correlation between the glucose and sorbitol concentrations (r = 0.563, P < 0.0001, n = 68), in agreement with the results of other investigators (1, 3, 7, 13). Scatter in the sorbitol/hemoglobin ratios for diabetic patients was expected because of the fluctuations in the glucose concentration. The sorbitol/hemoglobin values of normal patients, who had less glucose variation, were much more clustered (Figure 2).

Table 1. Validation of Standard Curve Results

<table>
<thead>
<tr>
<th>Sorbitol concn, μmol/L</th>
<th>Slope, mm vs μmol/L</th>
<th>Intercept, mm</th>
<th>Correlation, r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curve 1</td>
<td>11 1–15</td>
<td>7.49</td>
<td>-0.63</td>
</tr>
<tr>
<td>Curve 2</td>
<td>12 1–15</td>
<td>6.59</td>
<td>-0.23</td>
</tr>
<tr>
<td>Curve 3</td>
<td>12 2–15</td>
<td>9.70</td>
<td>-0.85</td>
</tr>
<tr>
<td>Curve 4</td>
<td>8 6–30</td>
<td>6.21</td>
<td>1.64</td>
</tr>
</tbody>
</table>

Table 2. Precision of the Assay

<table>
<thead>
<tr>
<th>Sorbitol concn, μmol/L</th>
<th>Mean #</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>15.1</td>
<td>1.84</td>
<td>12.2</td>
</tr>
<tr>
<td>6</td>
<td>46.1</td>
<td>1.57</td>
<td>3.41</td>
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<tr>
<td>6</td>
<td>59.2</td>
<td>2.79</td>
<td>4.72</td>
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<tr>
<td>10</td>
<td>65.0</td>
<td>1.75</td>
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</tr>
<tr>
<td>10</td>
<td>69.1</td>
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</tr>
<tr>
<td>10</td>
<td>70.8</td>
<td>2.07</td>
<td>2.93</td>
</tr>
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</table>

*Six replicates assayed 10 times each.
This improved procedure is accurate, precise, and sensitive for assaying sorbitol in human erythrocytes. Solutions and techniques were designed to minimize tedious manipulations and increase the speed of the assay. Solution concentrations were adjusted so that reagent additions in uniform volumes minimized dilution errors. Also, timing is not crucial in this assay, because the enzymatic reaction reaches a plateau in 20–25 min. This method is suitable for routine analysis and has been used to assay >1000 samples from the clinical study of an Alcon aldo reductase inhibitor. The effect of the inhibitor on sorbitol concentrations in diabetic patients has been reported elsewhere (16).

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References

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Liquid-Chromatographic Determination of Fleroxacin in Serum and Urine
Walid M. Awni, Jill A. Maloney, and Karen L. Helm-Duthoy

This highly sensitive, accurate, and reproducible HPLC method for determining fleroxacin in human serum and urine makes use of a common C18 column, a fluorescence detector, and an internal standard. Serum samples require a simple extraction procedure; urine must be diluted. The method, which we have used extensively for pharmacokinetic assessment of fleroxacin in patients, measures concentrations as low as 5 µg/L.

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Additional Keyphrases: antibiotics · pharmacokinetics · drug assay · chromatography, reversed-phase

Fleroxacin (R023-6240, AM-833) is a new quinolone derivative with broad in vitro antibacterial activity against Gram-positive and Gram-negative bacteria, similar to that of ofloxacin (1, 2). Of the few methods published for fleroxacin assay (3–5), one requires a gradient elution and takes about 35 min per run (3); another involves a high flow rate (3 ml/min), which is likely to shorten column life (5). All three have detection limits of ~100 µg/L. To investigate the pharmacokinetics of fleroxacin in humans, we have developed an HPLC method that is used with an isocratic mobile phase. A very simple extraction procedure is used for serum samples; urines require a simple dilution step but no extraction. The method has a 5 µg/L detection limit.