

An Enzymatic Method for Measuring Serum Mannitol and Its Use in Hemodialysis Patients

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We evaluated two chemical methods for quantifying mannitol in serum, based on the oxidation of mannitol by periodate, and measurement of the formaldehyde formed with chromotropic acid (colorimetry) or acetylacetone (fluorometry). We found interference in these methods by serum glycerol. Additionally, a high-performance liquid chromatography (HPLC) method was evaluated and found to be specific but impractical for routine use. We therefore, developed an enzymatic fluorometric procedure, based on the oxidation of mannitol by β -NAD to fructose and NADH, in the presence of the enzyme mannitol dehydrogenase (MD). MD is not commercially available and was partially purified from cultures of *Leuconostoc mesenteroides*. This new method is specific, sensitive, simple, and accurate and is proposed as the method of choice for measuring mannitol in the serum of patients who receive this sugar alcohol during routine hemodialysis treatment.

KEY WORDS: mannitol therapy; hemodialysis; enzymatic methods; fluorometric assay; mannitol dehydrogenase.

Introduction

Mannitol is an alcohol sugar not normally found in the human body. It is used to determine extracellular fluid volume, glomerular filtration rate, and as a marker of intestinal permeability (1-3). It is also used as an osmotically active agent in several clinical situations. It is especially useful during hemodialysis when its indications include prophylaxis and treatment of both hypotension and the dialysis disequilibrium syndrome (4). These two entities share a common pathophysiological disturbance that relates to the fact that dialysis can clear small osmotically active solutes from the blood faster than they can redistribute from the intracellular fluid compartment to the extracellular fluid compartment. This sets up an osmotic gradient for water movement from the extracellular space to the intracellular space. The dual mechanisms of loss of water

from the vascular space through ultrafiltration, and through osmotic forces into cells, predisposes to hypotension, while the movement of water into brain cells leads to the central nervous system symptoms of dialysis disequilibrium. Intravenous mannitol administration minimizes the shift of water to the intracellular fluid compartment (4).

When mannitol is given intravenously, it distributes throughout the extracellular fluid compartment. In individuals with normal renal function, approximately 80% is excreted in the urine and the remainder is eliminated slowly, probably through a hepatobiliary pathway (5). Massive accumulation of mannitol in patients with renal failure is known to be associated with an intoxication syndrome that includes encephalopathy, hyponatremia, a large osmolar gap, and fluid overload (5). It is thought that repeated mannitol administration during hemodialysis may lead to its accumulation. Whether chronic accumulation of mannitol causes toxicity is not known.

Despite widespread use of mannitol, the literature that guides the use of this agent is sparse and outdated. Dialysis has advanced significantly in the last 10 years. Dialysis schedules, dialysate flow-rates, blood flow-rates, dialysate composition, and mannitol doses and protocols for its administration have changed. Moreover, the methods used for the assay of mannitol in early pharmacokinetic studies were not specific.

We wished to estimate the clearance of mannitol during modern hemodialysis, the extent of mannitol accumulation in our present hemodialysis population, and the maximum safe limit of mannitol administration without accumulation. For these studies we need a reliable and specific method for mannitol assay in biological fluids. The early methods for mannitol assay rely on the oxidation of mannitol with periodate and measurement of formaldehyde by reaction with chromotropic acid [spectrophotometry (6,7)] or acetylacetone and ammonium acetate [fluorometry (8)]. These methods are subject to in-

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terference from glucose and glycerol. We have developed a new method that is based on the enzyme mannitol dehydrogenase and spectrofluorometry. The method has been used to measure mannitol in the serum of two patients who received the sugar alcohol during routine hemodialysis.

Materials and methods

REAGENTS

All solutions were made from analytical grade reagents. Methanol and acetonitrile were high performance liquid chromatography (HPLC) grade. All reagents were from Sigma Chemical Co., St. Louis, MO, USA, except when otherwise stated.

Solutions for the chromotropic acid method

87 mmol/L ZnSO₄; 83 mmol/L Ba(OH)₂; 20 mmol/L NaIO₄ in 0.5 mol/L H₂SO₄; 0.2 mol/L sodium arsenite. The last two reagents were prepared fresh on the day the assay was run. The chromotropic acid reagent was prepared by dissolving 1 g of chromotropic acid (Eastman Kodak, Rochester, NY, USA) in 100 mL H₂O, filtering it, and adding this solution to a mixture of 300 mL of 18 mol/L H₂SO₄ and 150 mL H₂O. This solution was found to be stable at 4 °C in a dark bottle for 2 months.

Solutions for the acetylacetone method

The same as for the chromotropic acid method; the acetylacetone reagent was prepared by adding 1.5 mL of acetylacetone and 5 mL isopropanol to 200 mL of a 2 mol/L ammonium acetate solution.

Solutions for the HPLC method

These were as described by Dethy *et al.* (9).

Solutions for the proposed enzymatic method

10% (w/v) trichloroacetic acid; Tris-HCl buffer, 1 mol/L, pH 9.0; β-NAD solution, 10 g/L in the previous Tris buffer; Tris-HCl buffer, 10 mmol/L, pH 8.6. Mannitol dehydrogenase solution was prepared as described below.

Mannitol standards

For all assays, we used mannitol standards prepared in normal human serum. The standards used were 0, 0.14, 0.27, 0.69, 1.37, and 2.75 mmol/L.

INSTRUMENTATION

All spectrophotometric measurements were carried out with the Shimadzu 160A double-beam UV-VIS spectrophotometer from Shimadzu Corp., Kyoto, Japan. All spectrofluorometric measurements were

carried out with the Shimadzu RF 5000 fluorimeter. The HPLC system used was also from Shimadzu. The column used for reverse-phase HPLC was a Supercar Cartridge column (C18) obtained from Pharmacia-LKB Canada, Dorval PQ. We used the 'Bead Beater' cell disrupting device from Biospec Products, Bartlesville, OK, USA.

PROCEDURES

Chromotropic acid method

We modified the procedure described in (7) to improve sensitivity. We used 100 μL serum instead of 50 μL and also used 600 μL of the protein-free filtrate instead of the recommended 300 μL. The final coloured product was measured at 570 nm.

Acetylacetone method

We used the method described in (8) for the measurement of the formaldehyde released after glycerol oxidation by NaIO₄. Serum (25 μL) was deproteinized with 500 μL each of ZnSO₄ and Ba(OH)₂ solutions. Two hundred μL of supernatant were mixed with 100 μL of 20 mmol/L sodium periodate solution. After 10 min, we added 100 μL of sodium arsenite solution and mixed. After 5 min we added 2 mL of acetylacetone reagent and mixed. Tubes were then incubated at 50 °C for 30 min. After cooling, we measured fluorescence with excitation and emission wavelengths of 410 and 513 nm, respectively.

HPLC method

We used the method of Dethy *et al.* (9) developed for sorbitol and galactitol. In this method, we deproteinized 400 μL of serum sample with 800 μL of methanol and evaporated 500 μL of the protein-free supernatant to dryness with a vacuum centrifuge (Emerston Instruments, Scarborough, ON, Canada). We then derivatized the residue with phenylisocyanate in the presence of pyridine at 55 °C as described (9). After derivatization, the samples were cooled on ice, and 250 μL methanol and 2 mL pyridine were added to each sample. Thirty μL of the resulting solution were injected into the HPLC system. The mobile phase was 60% acetonitrile, 40% 10 mmol/L K₂HPO₄, adjusted to pH 7.0 with H₃PO₄. The flow-rate was 1.2 mL/min; detection was at 237 nm. After each run, the column was washed with 100% acetonitrile for 2 min and reequilibrated for 8 min with the mobile phase before the new injection. Mannitol standards were prepared in serum and processed exactly as the samples.

Production and purification of mannitol dehydrogenase

We used a modification of the method of Lunn *et al.* (10) that is based on the work of Yamanaka (11). The growth medium contained 10 g/L peptone (Difco

Laboratories, Detroit, MI, USA), 2 g/L yeast extract (Difco), 0.12 mol/L sodium acetate, 0.9 mol/L magnesium sulfate, 17 $\mu\text{mol/L}$ sodium chloride, 9 $\mu\text{mol/L}$ manganese sulfate, and 56 mmol/L D-glucose.

Leuconostoc mesenteroides, ATCC 9135 was obtained from the American Type Culture Collection, Rockville, MD, USA. Other solutions and chemicals used were salmine sulfate, 20 g/L (BDH Inc., Toronto, Canada); potassium phosphate buffer, 50 mmol/L, containing 1 mmol/L mercaptoethanol, pH 7.0; ammonium sulfate and acetone.

The lyophilized powder of *Leuconostoc mesenteroides* was reconstituted with 8 mL growth medium and incubated at 30 °C overnight with shaking. This culture was then transferred to 400 mL of growth medium and incubated further for 24 h at 30 °C with shaking. The culture was then used to inoculate 10 L of growth medium that was incubated with aeration for 16–20 h at 30 °C with shaking. Cells were harvested by centrifugation and washed twice with 10 volumes of phosphate-mercaptoethanol buffer. The cells were resuspended in about 100 mL of the same buffer, cooled at 4 °C and disrupted with the 'Bead Beater' apparatus using an equal volume of glass beads as follows: 1 min blending followed by 1 min cooling. This was repeated four times. The crude extract was then used to isolate partially purified enzyme as described in detail by Lunn *et al.* (10). From the purification procedure we usually obtained about 10 mL of concentrated stock enzyme solution. After enzyme activity determination as described below, the enzyme solution is stored frozen at -20 °C as a ~10 U/mL solution in aliquots, until use.

Standardization of enzyme activity

The following procedure was used: in a 12 \times 75 mm tube add 50 μL of 0.55 mol/L mannitol aqueous solution, 1250 μL of β -NAD solution (10 g/L in Tris-HCl buffer, 100 mmol/L, pH 8.6), and 1–2 μL of enzyme solution. Mix well, transfer to a cuvette (1 cm pathlength) and monitor the absorbance change every 20 s, at 340 nm, over a period of 5 min, at room temperature. If dA/min is greater than 0.05 repeat the procedure with more dilute enzyme. One unit of enzyme is the amount required to generate 1 μmol of NADH per min under the assay conditions described. The formula used to calculate activity in U/mL is

$$\text{U/mL} = \frac{\text{dA/min}}{6.22} \times \frac{\text{volume of assay (mL)}}{\text{volume of enzyme sample (mL)}}$$

The stock enzyme solution is diluted to approximately 10 U/mL and used in the assay as described under the procedure.

Fluorometric assay procedure

In 1.5 mL microcentrifuge tubes, pipet 100 μL of serum samples or mannitol standards and 100 μL of

10% trichloroacetic acid solution, mix by vortexing, and let stand for 10 min at room temperature. Centrifuge at 12,000 $\times g$ for 5 min in a microcentrifuge. Set up 2 sets of 12 \times 75 mm tubes. The first (test set) consists of standards and the test samples. The second (blank set) consists of the zero standard only and all test samples. To each tube in the test set, add 40 μL protein free supernatant, 30 μL β -NAD solution (10 g/L in 1 mol/L Tris buffer, pH 9.0), 10 μL of enzyme solution (~10 U/ml) and mix. To each tube in the blank set add 40 μL of protein-free supernatant, 30 μL β -NAD solution, 10 μL Tris buffer 10 mmol/L, pH 8.6, and mix. Incubate all tubes at 37 °C for 1 h. Add 2.5 mL Tris buffer 10 mmol/L, pH 8.6 to all tubes, mix, and read fluorescence with excitation at 340 nm and emission at 450 nm.

The instrument is first zeroed with the zero mannitol standard containing enzyme and the fluorescence of all other standards and samples containing enzyme are read. Next, the instrument is zeroed with the blank zero standard (no enzyme added) and the fluorescence of the sample blanks are read. The sample blank readings are subtracted from the sample readings obtained in the presence of enzyme. A calibration curve is obtained by plotting the fluorescence of the standards *vs* the concentration of the standards and test results are obtained from this curve.

Results and discussion

EVALUATION OF THE CHROMOTROPIC ACID AND ACETYLACETONE METHODS

These two procedures are simple to perform, and utilize commercially available reagents and widely available instrumentation. Their detection limit was about 0.33 mmol/L and recoveries were between 97–103%. Linearity was up to 2.75 mmol/L and imprecision (1 CV) about 10% and 5–6% at mannitol levels of 0.33–0.69 and 0.69–2.75 mmol/L, respectively. Each 1 mmol/L glucose gave an apparent mannitol concentration of 0.01–0.02 mmol/L. Each 1 mmol/L glycerol reacted equivalently to 1.09 mmol/L mannitol.

We assayed mannitol in serum of 15 normal volunteers who had never received the sugar, by both methods, and found undetectable levels. However, when we tested sera from hemodialysis patients who had never received the sugar, we found significant levels of apparent mannitol concentration. For example, in five patients tested, the apparent mannitol concentration found by the acetylacetone method was (mmol/L): not detectable, 1.26, 0.07, 1.70, and 2.53, respectively. None of these patients had glucose greater than 10 mmol/L.

We analyzed glycerol concentrations in serum of both normal people and hemodialysis patients with an enzymatic procedure available from Boehringer Mannheim, Laval, PQ, Canada. No glycerol was found in normal sera. In the sera from the five hemodialysis patients previously mentioned, the levels

of glycerol found were (mmol/L): not detectable, 1.36, not detectable, 2.25, and 2.77 mmol/L. The correlation between measured glycerol levels and apparent mannitol levels as calculated by the chemical method has prompted us to suggest that glycerol is the major interferent in the chemical assays.

There are several reasons why glycerol may be present in the serum of hemodialysis patients. They usually have abnormal lipid metabolism and tri-

glyceride levels (12). In patients who receive heparin, an agent frequently used during hemodialysis, higher levels of serum glycerol are normally present due to triglyceride breakdown *in vivo* or *in vitro* (13,14). Other clinical conditions are associated with increases in serum glycerol (14). Because of this nonspecificity, the chemical methods for measuring mannitol in serum were considered unacceptable and not pursued further.

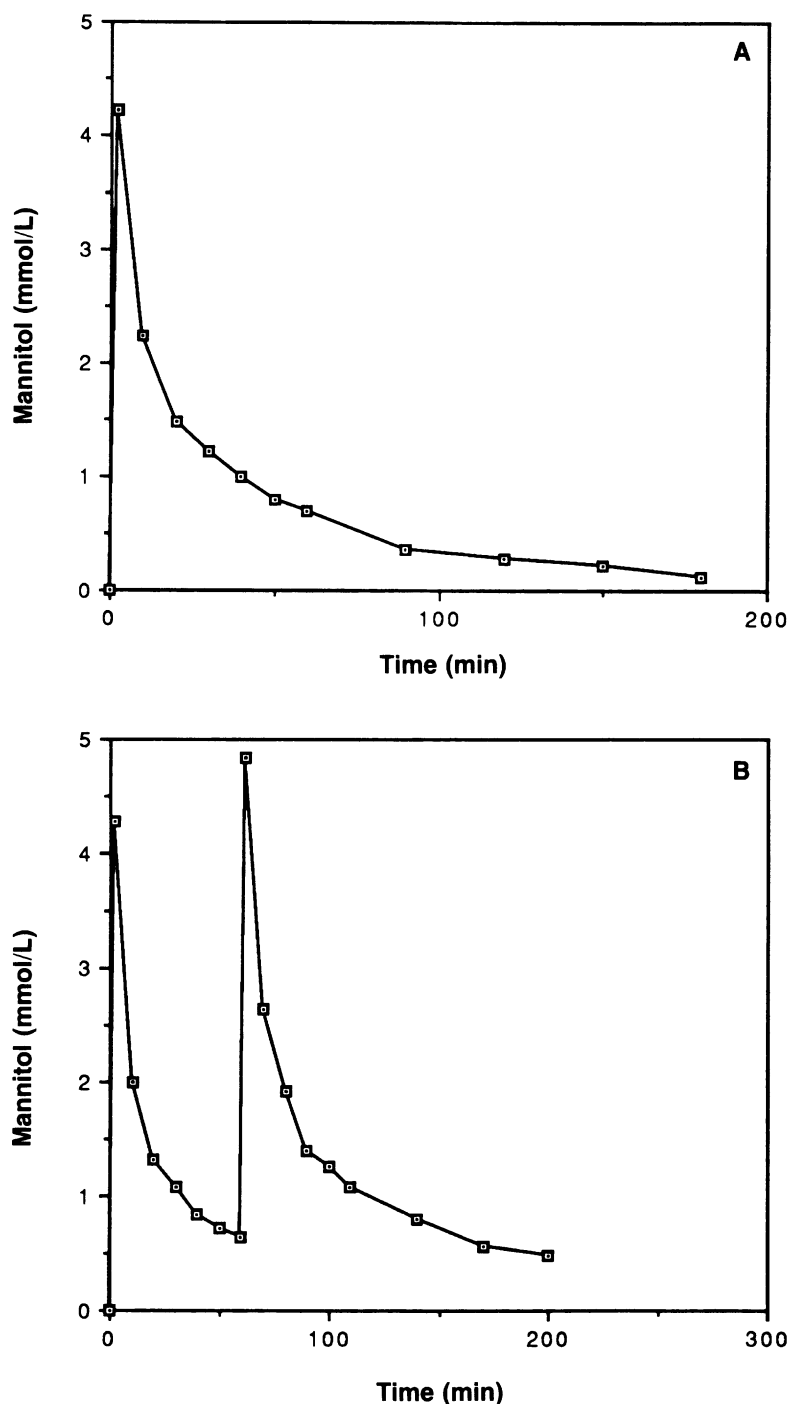


Figure 1 — Elimination of mannitol from serum of patients during hemodialysis. (A) Patient I received a mannitol bolus of 27.5 mmol (5 g) at time zero and mannitol levels were monitored at 2 min and regular intervals afterwards. (B) Patient II received two boluses of 27.5 mmol at time zero and 60 min.

EVALUATION OF AN HPLC METHOD

Dethy *et al.* (9) and others (15) have used chromatographic techniques to measure sugars in various types of specimen. We have adapted their method to measure mannitol in serum. After removing proteins with methanol, we derivatized the dried sample with phenylisocyanate to produce derivatives absorbing at 237 nm that could be separated by HPLC. With this method, the mannitol derivative elutes at ~27 min, as a single peak.

Recovery was found to be between 104–119% and linearity extends to 5.49 mmol/L. This method works well but has a number of disadvantages: it is lengthy, labour-intensive, and uses toxic chemicals and expensive instrumentation. If fully developed and evaluated, this assay could serve as a reference method but it is unsuitable for routine analysis of many samples per day.

DEVELOPMENT OF THE ENZYMATIC-FLUOROMETRIC PROCEDURE

The enzyme mannitol dehydrogenase (EC 1.1.1.67) is not available commercially, and in this study was produced following the procedure of Lunn *et al.* (10) that is based on previous work by Yamanaka (11). This enzyme catalyzes the conversion of mannitol, in the presence of β -NAD, to fructose and NADH. Lunn *et al.* described an automated procedure for measuring mannitol in urine by monitoring NADH absorbance at 340 nm (10) and Blomquist *et al.* (16) used a similar method to measure mannitol in dog serum. A procedure for human serum mannitol has not been published. We here describe a fluorometric assay for serum mannitol that uses small sample volumes, has high sensitivity and specificity, and requires low enzyme consumption.

A difficulty we encountered during enzyme preparation was the step of cell disruption to release enzyme. Repeated sonication as described by Lunn *et al.* (10) was not satisfactory. We found that lysis of cells by the 'Bead-beater' apparatus, which uses small glass beads colliding at high speed in the presence of the cells, was complete in minutes and we propose it as the method of choice for this application.

Mannitol dehydrogenase is very specific and many other sugars tested do not cross-react (10). We verified that our method for serum was specific by analysing samples from both normal individuals and hemodialysis patients who never received the drug and found undetectable levels in all cases. Samples included those that gave falsely positive results with the chemical methods. Furthermore, a study of glucose and glycerol interference with our method, showed that no cross-reactivity is seen at glucose and glycerol concentration in serum as high as 16 mmol/L.

With our method, fluorescence reaches a plateau at about 30–60 min and remains stable for at least

1 h thereafter. The working range extends to 2.75 mmol/L; the detection limit is around 0.05 mmol/L. Within-run CVs ($n = 21$) at levels of mannitol of 0.27, 0.82, and 2.75 mmol/L were 7.9%, 5.9%, and 4.7%, respectively. The day-to-day CVs ($n = 12$ over a period of 1 month) at various levels of mannitol (in parentheses) were 12.2% (0.14 mmol/L); 10.0% (0.27 mmol/L); 10.0% (0.69 mmol/L); 7.6% (1.37 mmol/L); and 8.0% (2.75 mmol/L). Analytical recovery of added mannitol in serum ranged from 82–100% with a mean of 95%.

CLINICAL APPLICATION

We have used this assay to monitor the levels of mannitol in the serum of two patients who received the sugar alcohol during routine hemodialysis. The results are presented graphically in Figure 1.

To optimize administration protocols and avoid possible acute and chronic toxicity in these patients, it is necessary to have a means of monitoring mannitol levels. We have demonstrated that in this group of patients, the chemical methods, widely used in the past, are not reliable because they suffer from glycerol interference. While methods based on HPLC could offer an accurate alternative, these methods are slow and time consuming. They also use toxic chemicals and expensive instrumentation. The method that we developed is sensitive, specific, and simple, permitting analysis of many samples within a working day. We propose this procedure as the method of choice for monitoring mannitol levels in serum. We are currently using this method to monitor mannitol levels during and after hemodialysis, to derive pharmacokinetic parameters, to adjust doses, and to assess chronic toxicity in hemodialysis patients.

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Note added in proof: Mannitol dehydrogenase has just become commercially available from Sigma Chemical Co., St. Louis, MO, USA.

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