Automated Vitamin C Analysis

Hans U. Aeschbacher and R. Glenn Brown

An automated method of analysis for vitamin C (ascorbic acid, dehydroascorbic acid, diketogulonic acid) is described, in which ascorbic acid as oxidized to dehydroascorbic acid with Chloramine-T and determined by reaction with 2,4-dinitrophenylhydrazine. Results obtained by automated and manual methods agreed within 0.89% for ascorbic acid in liver. By the automated method, 96.7% of ascorbic acid added to tissue homogenates could be accounted for. The principal drawback to the proposed method is the use of 70% perchloric acid, a potent oxidizing agent, to solubilize the osazone.

Additional Keyphrases 2,4-dinitrophenylhydrazine  • AutoAnalyzer  • ascorbic acid, dehydroascorbic acid, diketogulonic acid  • perchloric acid oxidant  • Chloramine-T

Several manual methods exist for determining vitamin C in various substances. Two methods commonly used are the 2,6-dichlorophenolindophenol reduction technique (1) and the 2,4-dinitrophenylhydrazine condensation method (2). The first technique has been automated (3), but metal ions interfere with both the manual and automated methods. Because the 2,4-dinitrophenylhydrazine method is not particularly sensitive to such interference, it is satisfactory for most routine use, although it is not as specific for ascorbic acid (4) as is the dye-reduction technique. It is a rather slow technique, principally because of the charcoal oxidation and subsequent filtration step.

We undertook to automate the 2,4-dinitrophenylhydrazine technique for use with a single-channel AutoAnalyzer.

Materials and Methods

Reagents and Apparatus

Chloramine-T. Chloramine-T (Eastman Organic Chemicals, Rochester, N.Y.), 0.5 g, was dissolved in 80 ml water and diluted with 120 ml of 2-methoxyethanol (British Drug Houses, Rexdale, Ontario) and 200 ml of buffer. This solution must be made daily.

Buffer. This consists of 50 g of citric acid monohydrate, 12 ml of glacial acetic acid, 120 g of sodium acetate (3 H2O), 34 g of sodium hydroxide, diluted with water to 1 liter. The pH is adjusted to pH 6, and the finished buffer should be stored in the cold under toluene.

Thiourea. Dissolve 10 g of thiourea in 100 ml of ethyl alcohol:water (1:1, by vol). This reagent is not stable for more than two months.

2,4-Dinitrophenylhydrazine reagent. Dissolve 10 g of 2,4-dinitrophenylhydrazine in 500 ml of sulfuric acid (4.5 mol/liter). Filter if necessary and store in a dark glass bottle.

Perchloric acid. Analytical grade 70% perchloric acid was used. Perchloric acid is a potent oxidizing agent; it must be handled with care and kept in a spill-proof glass container.

Standards. A stock solution of 100 mg of L-ascorbic acid per 100 ml of distilled water was prepared and kept frozen in small lots. Standards are prepared from this stock solution containing 1, 2.5, 5, and 10 mg/100 ml. These standards should be prepared daily and kept chilled until used.

Apparatus. A single-channel AutoAnalyzer (Technicon, Ltd., Montreal, P.Q.) was used for these determinations (manifold diagram is shown in Figure 1). Solvaflex pump tubing was used to carry the thiourea reagent and Aciflex pump tubing was used for the 2,4-dinitrophenylhydrazine reagent, the 70% perchloric acid reagent, and the waste. Aciflex tubing was also used to transport the fluid after the point at which the 2,4-dinitrophenylhydrazine reagent was added. Conven-

Fig. 1. Diagram of AutoAnalyzer manifold for automated vitamin C analysis

From the Department of Animal and Poultry Science, University of Guelph, Guelph, Ontario, Canada.

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1 Present address: Société d'Assistance Technique, Nestlé, S. A., Vaud, Switzerland.
tional transmission tubing should not be used because it deteriorates rapidly. The pick-up tube was a No. 14 stainless-steel hypodermic needle, which was used to aspirate a sufficiently large sample. The sampler was operated at 60 samples per hour with a 2:1 wash:sample ratio.

Preparation of Samples

Fresh tissue (liver) was homogenized in the cold in sufficient trichloroacetic acid (TCA) solution (60 g/liter) that the final mixture contained 20 g of fresh tissue per 100 ml. The homogenate was either filtered or centrifuged (5,000 × g, 2°C) and the clear supernatant fluid was analyzed, without further dilution, by both the automated technique and the manual technique (3). In addition, recovery was measured in a separate series of experiments.

Procedure

The final procedure is illustrated in Figure 1. To get complete separation of the peaks, it was necessary to place two sample cups of water between each sample cup. A surfactant, “Brij-35” (Technicon), 0.5 ml/liter, was added to the water, to maintain an easy flow of reagents through the lines. The lines should be well flushed with hydrochloric acid (6 mol/liter), followed by water, at the end of the day’s operation. Failure to do this will result in coils and junctions being clogged by precipitated crystalline materials, which probably are osazones.

Results and Discussion

Figure 2 shows a typical standard curve for L-ascorbic acid. The response was such that a reasonably steep curve was obtained, which would allow for good sensitivity. The coefficient of variation about the points on standard curves in use in our laboratory was 6%. When the percent transmission values were converted to absorbance, a straight-line relationship between concentration and absorbance was found for concentrations up to 10 mg/100 ml, which suggests that Beer’s law is followed in the range of physiological fluids. If the sampling volume was halved, Beer’s law applied up to concentrations of 25 mg/100 ml, although there was a loss of sensitivity at the lower concentrations. In Table 1 tissue analyses by the automated method and the manual method are compared, and show the good agreement (difference, 0.89%) between the two methods in terms of amounts found. For 10 samples recovery of added L-ascorbic acid averaged 96.7% (range, 90–102%) by the proposed method.

For obvious reasons “Norite” charcoal was not a satisfactory oxidizing agent for the automated procedure. Hydrogen peroxide, sodium hypochlorite, and Chloramine-T were tested as oxidizing agents, but only Chloramine-T produced constant results with standard solutions. The optimal concentration of Chloramine-T was 0.50 g/400 ml of solution. When 0.25 g/400 ml solution was used, no response was observed; if 0.75 g/400 ml of solution was used, a heavy white precipitate formed when the 2,4-dinitrophenyl-hydrazine reagent was added that completely blocked the flow. The results obtained when
Chloramine-T was used as the oxidizing agent were identical to those found with Norite as an oxidizing agent (Table 2).

The osazone formed with 2,4-dinitrophenylhydrazine was solubilized by Roe and Kuether (2) with 85% sulfuric acid without the removal of excess 2,4-dinitrophenylhydrazine reagent. Such a solution is too viscous to flow satisfactorily in the automated system. Other solvents we tried—such as acetic acid, hydrochloric acid, formic acid, and phosphoric acid—gave indifferent results; either the solution became turbid, the osazone was not dissolved, or spurious colors were formed. The solvent that produced a nonviscous solution without unwanted side reactions was 70% perchloric acid. There was a very low heat of solution generated when perchloric acid was used as a solvent and the ice bath required when sulfuric acid was used could be replaced with a single cooling coil. We saw no charring of sugars or organic material when perchloric acid was used. It should be remembered that perchloric acid is a potent oxidizing agent and extreme care must be exercised in handling it. We used a small glass container set in a large leaden base to avoid accidental spills.

Because of the very small diameter of the pump tubing for both Chloramine-T and thiourea reagents, some time is required for these reagents to reach the fluid stream, and one must make sure that these reagents are present in the system before sampling is started or the baseline is established.

The automated method described above has been found to be satisfactory for analysis of simple solutions containing vitamin C or for routine analysis of tissue extracts. Because the 2,4-dinitrophenylhydrazine method is not as selective as the dye-reduction techniques, it is more appropriate if total vitamin C (ascorbic acid, dehydroascorbic acid, and diketogulonic acid) analysis is desired.

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References


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<tr>
<th>L-Ascorbic acid</th>
<th>Norite Absorbance, 540 nm</th>
<th>Chloramine-T Absorbance, 540 nm</th>
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*Comparison made using manual method in which 1 ml of Chloramine-T solution was used to oxidize 4 ml of standard solution. In the Norite oxidized sample, 1 ml of Chloramine-T solution was added to 4 ml of Norite filtrate. All readings were made on a Unicam SP800 recording spectrophotometer.*