Oxidation of Urinary Ascorbic Acid by Ascorbate Oxidase Immobilized onto a Test Tube Wall

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SUMMARY Ascorbic acid is well known to give false negative results in the clinical determination of urinary glucose or occult blood (blood/hemoglobin) using commercially available reagent test strips. We describe herein a procedure to remove the ascorbic acid interference with use of a newly deviced enzyme reactor, where ascorbate oxidase is immobilized on the inner surface of polystyrene test tubes treated with a silane coupling reagent. It was clearly shown that even the highest possible concentration of ascorbic acid in urine can be completely oxidized within a few minutes at room temperature by the enzyme reactor, and therefore urinary glucose or occult blood can be determined without any false negative results. This type of enzyme reactor may be applicable for use in many clinical fields and in many testing situations in clinical laboratories to improve the accuracy and reliability of clinical tests.

Introduction

Reagent test strips are widely used in clinical laboratories for the qualitative or semi-quantitative determination of urinary glucose or occult blood. However, the trouble is that ascorbic acid interferes with the peroxidase-linked hydrogen peroxide determination to give false negative results in the determination of glucose or occult blood1,2. Although ascorbate oxidase (AOD) from Cucumis, which catalyzes the oxidation of ascorbic acid to dehydro-ascorbic acid and water, is generally used to remove the interference with ascorbic acid, it is not economical to use an aqueous enzyme solution on each sample; also, the presence of the enzyme on the test strips may have some undesirable effects on the determination.

The present study was undertaken to prepare an AOD-immobilized test tube (AOD reactor) and to use it for the determination of urinary glucose or occult blood without any interference from ascorbic acid.
Materials and Methods

Materials

1. $\gamma$-aminopropyltriethoxysilane solution (Shin-Etsu Chemical Co., Ltd.)
2. Glutaraldehyde solution, 12.5%: Mix 50 ml of glutaraldehyde solution (25%, Yoneyama Yakuhin Kogyo Co., Ltd.) with an equal volume of water.
3. Ascorbate oxidase (AOD) solution, 200 units/ml: Dissolve 10 mg of lyophilized ascorbate oxidase (from Cucumis sp., Toyobo Co., Ltd.) in 10 ml of water.
4. Ascorbic acid solution, 1, 5, or 10 mmol/l: Dissolve 176 mg of L-ascorbic acid (Wako Pure Chemical Industries, Ltd.) in water, and make up to 100 ml with water (10 mmol/l ascorbic acid solution), and dilute either 2 or 10-fold (5 mmol/l or 1 mmol/l ascorbic acid solution, respectively) in water.
5. Phosphotungstate reagent$: Solution A$, to a mixture of 201 g of disodium hydrogen phosphate 12-hydrate and 200 g of sodium tungstate, add 200 ml of water and warm to dissolve. Solution $B$, add 50 ml of sulfuric acid (spec. gravity 1.84) to 150 ml of water. Pour solution $B$ into solution $A$, and boil the mixture gently for 2 hours under reflux.
6. Phosphate buffer, 0.1 mol/l, pH 7.0: Dissolve 21.9 g of sodium phosphate, dibasic, 12-hydrate and 6.1 g sodium phosphate, monobasic, 2-hydrate in water, and make up to 1000 ml with water. Check pH with a pH meter and adjust accurately if necessary.
7. Polystyrene test tube: “Serapittzu EI” (11φ ×72 mm, Ono Pharmaceutical Co., Ltd.)
8. Reagent test strips: “Rapignost Total-

Preparation of AOD reactor

Immobilization of AOD onto the wall of a polystyrene test tube was performed by a modification of Kosaka’s procedure$^4$.

The tube was filled with $\gamma$-aminopropyltriethoxysilane solution and allowed to stand for 10 min at room temperature, followed by washing with water. Then, the tube was filled with 12.5% glutaraldehyde solution and allowed to stand for 20 min in an ice bath, followed by a thorough washing with 0.1 mol/l phosphate buffer (pH 7.0).

Lastly the tube was again filled out with AOD solution (200 units/ml), kept for 12 hours at 4°C, and then thoroughly washed with the buffer.

Test tubes prepared as described above were kept in 0.1 mol/l phosphate buffer (pH 7.0) at 4°C until used.

The enzyme activity of AOD reactors was between 6.0–9.0 units/tube.

Oxidation of ascorbic acid with the AOD reactor

One milliliter of ascorbic acid solution (1, 5, or 10 mmol/l) was added to the AOD reactor. Then the solution was stirred for 0 to 10 min by a Coulter mixer (Coulter Electronics Ltd.) at room temperature (called “sample X”).

Determination of ascorbic acid

A 0.05 ml volume of sample X was mixed with 3 ml of phosphotungstate reagent, and the mixture was allowed to stand for 15 min at 37°C. Absorbance at 700 nm was read against a reagent blank. The calibration curve was linear up to 10 mmol/l.

Screen A" (Behring Institut)
Results

Enzymatic oxidation of various concentrations of ascorbic acid

The enzymatic oxidation of various concentrations of ascorbic acid (1, 5, or 10 mmol/l) by the AOD reactor (9.0 units/tube) is shown in Fig. 1. Approximately 95% of the ascorbic acid in the 1 or 5 mmol/l solution was oxidized within 2 min, and the 10 mmol/l solution was completely oxidized in about 6 min. The difference in AOD activity (6.0-9.0 units/tube) among individual AOD reactors did not practically affect the oxidation time of ascorbic acid at any of the three concentrations tested.

Effect of pH

Enzymatic oxidation of ascorbic acid at different pH's (pH 5–pH 8) was investigated. As shown in Fig. 2, the oxidation under mildly acidic conditions (pH 5–6) was slightly faster than that under a mildly alkaline one (pH 8). However, the influence of a difference of the three pH (from 5 to 8) on the oxidation time of ascorbic acid was scarcely recognizable.

Stability of AOD reactor

The functional stability of the AOD reactor was investigated over a 90-day period. As shown in Fig. 3, the reactor was fully stable for 36 days.

Frequency of ascorbic acid-contaminated urine

To find ascorbic acid contamination of the urine, the reagent test strips, “Rapignost Total-Screen A”, which contain an ascorbic acid indicator, were used. Out of urine samples from 500 patients,
The AOD reactors were stored in 0.1 mol/l phosphate buffer (pH 7.0) at 4°C for 1-90 days. On the indicated days different concentrations of ascorbic acid (△—△, 1 mmol/l; ○—○, 5 mmol/l; ●—●, 10 mmol/l), 1 ml, were oxidized in the AOD reactors at 26°C, and the time required for the complete oxidation was determined.

74 urines were found to be ascorbic acid contaminated (>7 mg/dl), which amounted to 14.8% of all samples.

**Application of AOD reactor to urinary samples**

To determine the effectiveness of the AOD reactor, urine samples which were measured as ascorbic acid-positive and glucose- or occult blood-negative by the "Rapignost Total-Screen A" were tested after treatment with the AOD reactor.

Out of 185 patients' urines, which were measured as glucose-negative but ascorbic acid-positive, 37 urines (20.0%) were found to be glucose-positive after treatment with the AOD reactor; and 18 urines (8.5%) out of 211 urines were measured as occult blood-positive after being treated with the AOD reactor, as shown in Fig. 4(a) and Fig. 4(b)

The results described above clearly show that newly deviced AOD reactor eliminates misleading false negative results.

**Discussion**

Ascorbic acid, a strong reductant, is well known to interfere with peroxidase-dependent indicator systems by the breakdown of hydrogen peroxide or the reductive reversal of dye colour. In the determination of urinary glucose or occult blood
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Using qualitative or semi-quantitative reagent test strips, false negative results are often given in the presence of ascorbic acid. The use of AOD is probably the most effective method to eliminate the interference by ascorbic acid. However, it is not economical to use aqueous AOD for the elimination of the ascorbic acid interference in these qualitative tests.

In the present study, AOD-immobilized polystyrene test tubes were prepared for repeated use as an enzyme reactor and applied for the enzymatic removal of contaminating ascorbic acid in urine. The AOD reactor thus formed was shown to catalyze the rapid oxidation of ascorbic acid in urine. Even the highest possible concentration of ascorbic acid was practically oxidized within a few minutes. A single AOD reactor has been used for more than 200 determinations for at least for a month without a significant loss of activity. Ascorbic acid was usually oxidized between pH 5-8, and no significant differences among reactors were noted.

Pretreatment of ascorbic acid-positive urine with the AOD reactor greatly reduced the frequency of false negative results in the determination of urinary glucose and occult blood: nearly 20% of urine samples being read as glucose-negative but ascorbic acid-positive were confirmed to be glucose-positive after removal of ascorbic acid with the AOD reactor.

This type of enzyme reactor should prove to be quite useful in many laboratory examinations to improve the accuracy and reliability of clinical tests, which are susceptible to interference by ascorbic acid.

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References

4) A. Kosaka : Rinsho Byouri, 24 (suppl) 202 (1976) in Japanese