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DETERMINATION OF ASCORBIC ACID IN HUMAN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH FLUORIMETRY AFTER POST-COLUMN DERIVATIZATION WITH BENZAMIDINE

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SUMMARY

High-performance liquid chromatography on two Asahipak GS-320 hydrophilic gel columns (50 × 0.76 cm I.D.), connected in series, with 0.015 *M* tartrate buffer (pH 3.0), containing 2 m*M* ethylenediaminetetraacetate and 0.05% β-thiodiglycol as eluent allowed the separation of glucose, diketogulonic acid + diketogluconic acid, dehydroisoascorbic acid, dehydroascorbic acid, ascorbic acid, and isoascorbic acid within 55 min. Ascorbic acid in a urine sample was stabilized by the addition of an equal volume of 5% metaphosphoric acid solution, containing 0.5% of β-thiodiglycol. Filtration of the mixture through a column of Dowex 50W-X8 (H^+) facilitated the determination of ascorbic acid and isoascorbic acid in human urine. Samples could be analyzed every 20 min.

INTRODUCTION

Urinary levels of ascorbic acid are said to reflect recent dietary intakes[1]. However, the instability of ascorbic acid and the presence of interfering substances in human urine has prevented routine determination. Photometric methods, based on the reducing power of ascorbic acid or on the formation of the dinitrophenylhydrazine derivative of dehydroascorbic acid lack sensitivity[1,2]. Methods based on high-performance liquid chromatography (HPLC) provide good separation of ascorbic acid from isoascorbic acid[3–5], but UV or electrochemical detection yields chromatograms with some interfering peaks[6,7]. Both pre- and post-column derivatization with fluorometric detection improve the sensitivity[7–10], but application of this method to the determination of urinary ascorbic acid has not yet been reported.

In a previous report we have shown that ascorbic acid, dehydroascorbic acid, diketogulonic acid, and glucose could be separated on a column of a hydrophilic gel, Asahipak GS-320, by isocratic elution, using an aqueous buffer of pH 3.0. This

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method was applied for the determination of ascorbic acid in fruit juice[11]. Better resolution of ascorbic acid from isoascorbic acid is obtained by using two columns of Asahipak GS-320, connected in series. Pretreatment of urine by use of a column of sulphonated polystyrene resin in the hydrogen ion form permits the determination of urinary ascorbic acid and isoascorbic acid free from interference by fluorescent impurities.

EXPERIMENTAL

Materials and solutions

Benzamidine hydrochloride was a product of Tokyo Kasei Kogyo (Tokyo, Japan), potassium sulphite (reagent grade), and other chemicals (analytical grade); were purchased from Yashima Pharmaceutrical (Osaka, Japan). Water (ultrapure grade) was prepared by reverse osmosis (ROpure 40; Barnstead, Boston, MA, U.S.A.), ion exchange, and charcoal adsorption of organic matter (NANOpure-II; Barnstead).

The mobile phase was prepared by dissolving 4.5 g of tartaric acid, 1.5 g of disodium ethylenediaminetetraacetate and 1 g of β-thiodiglycol in 2 l of ultrapure water and adjusting the pH to 3.00–3.03 with 4 *M* sodium hydroxide solution. The solution was filtered through a membrane filter (pore size 0.45 μm) and degassed under vacuum before use.

The concentration of the primary stock solutions of ascorbic acid and isoascorbic acid was 1 m*M* in the mobile phase. A secondary standard was prepared by diluting the stock solution with the mobile phase to give a solution of 20 μ*M*. These standard solutions were prepared daily.

Dehydroascorbic acid was prepared by oxidation of ascorbic acid with iodine solution. The iodine solution was prepared by dissolving 12.7 mg of iodine in a concentrated solution of 25 mg of potassium iodide in ultrapure water and then diluting the solution to 100 ml with ultrapure water. A volume of 1 ml of the solution of ascorbic acid in water (2 m*M*) was mixed with 6 ml of the iodine solution and, after 1 min at 20°C, the excess iodine was destroyed by the addition of three drops of β-thiodiglycol. This solution was diluted with the mobile phase to give a solution of 8 μ*M* which was analyzed immediately. Dehydroisoascorbic acid was prepared by oxidation of isoascorbic acid using the procedure described above.

Diketogulonic acid and diketogluconic acid were prepared by hydrolysis of dehydroascorbic acid and dehydroisoascorbic acid, respectively. A solution of oxidized ascorbic acid or isoascorbic acid, described above, was neutralized with 100 μl of 0.5 *M* sodium hydrogen carbonate solution and incubated for 1 h at 30°C. The solution was diluted to 50 ml with the mobile phase, and 5 ml of the solution was diluted again with the mobile phase to 50 ml to give a solution of 4 μ*M*.

Reagent A was a solution of benzamidine hydrochloride (0.02 *M*) and reagent B was potassium borate buffer of pH 10 (0.75 *M*), containing 0.2 *M* potassium sulphite.

Equipment

The chromatographic system consisted of a constant-delivery pump (Tri Rotar-III; Jasco, Tokyo, Japan), an automatic sampler (Model KSST-60J; Kyowa Seim-

itsu, Tokyo, Japan) and two columns of hydrophilic gel (Asahipak GS-320, 50 × 0.76 cm I.D.; Asahi Chemical Industry, Japan), connected in series. A dual-head pump (Model SP-024-2; Jasco) was used to pump the reagents and mix them with the effluent from the column. A Model FP-550F spectrofluorometer (Jasco), equipped with a flow-cell (7 μl) was used to monitor the fluorescence.

Preparation of Dowex 50 column

Dowex 50W-X8, 200–400 mesh, was suspended in water and poured into a chromatographic tube (10 × 0.7 cm I.D.; equipped with a 10-ml reservoir) and allowed to settle under gravity to form a resin bed, 2.5 cm high. Then it was washed with 10 ml of 2 *M* hydrochloric acid solution, followed by deionized water until the washings became neutral.

Pretreatment of human urine

Fresh human urine was mixed with an equal volume of 5% (w/v) metaphosphoric acid solution, containing 0.5% (w/v) β-thiodiglycol, and stored in an ice-bath or in a refrigerator at −1°C. A volume of 1 ml of the mixture was added to the Dowex 50 column, inserted in a test tube (15 × 1.8 cm O.D.), containing 50 μl of 5% (w/v) disodium ethylenediaminetetraacetate solution and immersed in an ice-bath. After the sample had penetrated the column, the column was washed with 3.95 ml of 2 m*M* tartaric acid solution, containing 0.05% β-thiodiglycol. The eluate and washings in the test tube were filtered through a disposable filter unit (ED-03, pore size 0.45 μm; Gelman Sciences Japan, Tokyo, Japan) and 50–250 μl of the filtrate was used for analysis.

Chromatographic separation and fluorometric determination

A volume of 50–250 μl of standard or sample solution was injected into an Asahipak GS-320 column, maintained at 30°C. The mobile phase was pumped at a flow-rate of 1.0 ml/min and the eluate was mixed, using a T-shaped connector, with a 1:1 mixture of reagents A and B. Reagents A and B were pumped with a dual-head pump at a flow-rate of 0.36 ml/min each and mixed, using the T-shaped connector. The mixture was heated at 90°C in a polytetrafluoroethylene tube (50 m × 0.5 mm I.D. × 1.5 mm O.D.) immersed in a water bath. Fluorescence was measured with excitation at 325 nm and emission at 400 nm.

RESULTS AND DISCUSSION

As shown in Fig. 1, glucose, diketogulonic acid, dehydroisoascorbic acid, dehydroascorbic acid, ascorbic acid and isoascorbic acid were eluted in that order within 55 min. Diketogulonic acid overlapped with diketogluconic acid. Since ascorbic acid is easily oxidized in aqueous solution, it was slowly converted to dehydroascorbic acid during chromatography on Asahipak GS-320. Dehydroascorbic acid appeared as a low plateau ahead of the peak of ascorbic acid as shown in Fig. 2. Assuming that all the dehydroascorbic acid in the plateau resulted from ascorbic acid during chromatography, the relative conversion of ascorbic acid to dehydroascorbic acid was calculated as 2.5% when 150 μl of 20 μ*M* ascorbic acid solution was injected, and 6% when 4 μ*M* ascorbic acid was injected. Thus, as the amount of ascorbic acid

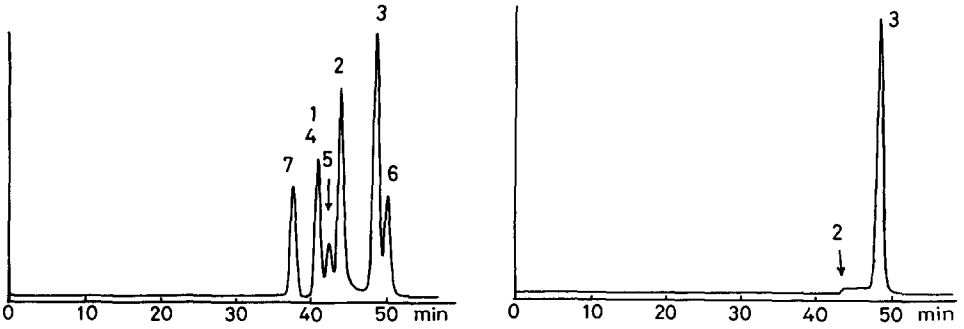

Fig. 1. Elution pattern of glucose, diketogulonic acid, dehydroascorbic acid, ascorbic acid, diketogluconic acid, dehydroisoascorbic acid, and isoascorbic acid. Peaks: 1 = diketogulonic acid (4 μM); 2 = dehydroascorbic acid (8 μM); 3 = ascorbic acid (20 μM); 1 and 4 = diketogulonic acid and diketogluconic acid (4 μM each); 5 = dehydroisoascorbic acid (8 μM); 6 = isoascorbic acid (20 μM); 7 = glucose (1 mM). The volume of sample injected was 150 μl. For chromatographic conditions see text.

Fig. 2. Elution pattern of ascorbic acid (20 μM). Numbers in the figure are the same as those indicated in Fig. 1. The volume of sample injected was 150 μl. The low plateau in front of the peak of ascorbic acid is dehydroascorbic acid, formed from ascorbic acid inside the column.

injected into the column decreased, conversion to dehydroascorbic acid increased, but at each concentration, the peak height of ascorbic acid was quite reproducible, *i.e.*, the coefficient of variation was 2.5% for the former and 3.1% for the latter, and working curves showing the relationship between peak height and concentration of the sample could be drawn. With isoascorbic acid, the relative conversion to dehydroisoascorbic acid was slightly less than that of ascorbic acid to dehydroascorbic acid. Since isoascorbic acid is eluted after ascorbic acid, it will be necessary to correct the peak height of ascorbic acid for the presence of dehydroisoascorbic acid when isoascorbic acid is present in a sample. The increase in the peak height of ascorbic acid in such a case will be 1 or 2% of the peak height of isoascorbic acid for a sample containing 40 μM or 8 μM isoascorbic acid, respectively.

A sample of dehydroascorbic acid, prepared by iodine oxidation of ascorbic acid, contained about 1% of diketogulonic acid. Since the coefficient of variation of the peak height of dehydroascorbic acid was larger than that of ascorbic acid and isoascorbic acid under the present conditions, we did not attempt to determine dehydroascorbic acid in this work.

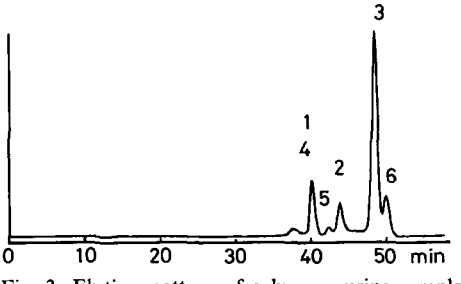

Fig. 3. Elution pattern of a human urine sample, filtered through a column of Dowex 50W-X8. The volume of sample injected was 200 μl. Numbers in the figure are the same as those indicated in Fig. 1.

We stabilized ascorbic acid in urine by addition of an equal volume of 5% metaphosphoric acid solution, containing 0.5% of β-thiodiglycol. After the mixture was kept in an ice bath for 20 h, the change in the content of ascorbic acid was less then 3%.

The elution pattern of a human urine sample is shown in Fig. 3. The retention times of peaks 2, 3, 5 and 6 correspond to those of dehydroascorbic acid, ascorbic acid, dehydroisoascorbic acid and isoascorbic acid. Although the retention time of peaks 1 and 4 are shorter than those of diketogulonic acid and diketogluconic acid by 0.6 min, addition of diketogulonic acid to the sample indicated that peak 1 and 4 corresponds to the peak of diketogulonic acid and diketogluconic acid. Samples could be analyzed every 20 min, and the recovery of ascorbic acid and isoascorbic acid added to a urine sample at concentrations of 10 μM and 50 μM, respectively, were 98.2 ± 1.7% and 98 ± 1.5% (S.D., five determinations).

The results indicate that Asahipak GS-320, a cross-linked vinyl alcohol copolymer, is suitable for the estimation of ascorbic acid and isoascorbic acid in human urine.

REFERENCES

1 S. T. Omaye, J. D. Turnbull and H. E. Sauberlich, *Methods Enzymol.*, 62 (1979) 3.
2 D. Nagahara, *Rinsho Byori*, 31 (1983) 259.
3 J. Geigert, D. S. Hirano and S. L. Neidleman, *J. Chromatogr.*, 206 (1981) 396.
4 L. W. Doner and K. B. Hicks, *Anal. Biochem.*, 115 (1981) 225.
5 J. M. Coustard and G. Sudraub, *J. Chromatogr.*, 219 (1981) 338.
6 C. S. Taso and S. L. Salimi, *J. Chromatogr.*, 245 (1982) 355.
7 W. O. Mason, E. N. Amick and W. Heft, *Anal. Lett.*, 13 (B10) (1980) 818.
8 A. J. Speek, J. Schrijver and W. H. P. Schreurs, *J. Chromatogr.*, 305 (1984) 53.
9 T. Iwata, M. Yamaguchi, S. Hara, M. Nakamura and Y. Ohkura, *J. Chromatogr.*, 344 (1985) 351.
10 J. T. Vanderslice and D. J. Higgs, *J. Chromatogr. Sci.*, 22 (1984) 485.
11 T. Seki, Y. Yamaguchi, K. Noguchi and Y. Yanagihara, *J. Chromatogr.*, 332 (1985) 283.