Journal of Chromatography, 332 (1985) 283–286
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 619

SEPARATION OF ASCORBIC ACID, DEHYDROASCORBIC ACID, DIKETOGULONIC ACID AND GLUCOSE BY ISOCRATIC ELUTION FROM A COLUMN OF A HYDROPHILIC GEL

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(Received April 6, 1985)

SUMMARY

High-performance liquid chromatography on an Asahipak GS-320 hydrophilic gel column with tartrate buffer (0.015 *M*, pH 3.0) containing 2 m*M* ethylenediaminetetraacetate and 0.05% β-thiodiglycol as the eluent allowed the separation of glucose, diketogulonic acid, dehydroascorbic acid and ascorbic acid within 30 min. Fluorimetric monitoring of these compounds in the eluate with benzamidine at alkaline pH and at 90°C in the presence of potassium sulphite allowed the determination of nanogram amounts of ascorbic acid, dehydroascorbic acid and diketogulonic acid. This method was applied to the determination of ascorbic acid in fruit juice.

INTRODUCTION

The separation of ascorbic acid, dehydroascorbic acid, diketogulonic acid and their C-5 epimers has been performed by ion-exchange chromatography on a weakly basic ion exchanger using acetonitrile–0.05 *M* potassium dihydrogen phosphate solution (3:1, v/v) as eluent[1] and a mobile phase containing less organic component was used for the determination of ascorbic acid and isoascorbic acid in fruit juice[2]. The separation of these compounds was also possible by ion-pair chromatography on a reversed-phase column[3,4]. Ascorbic acid in eluates has been detected electrochemically[5,6] and from its characteristic absorption at 265 nm[1–4]. The formation of a fluorophor by condensation of dehydroascorbic acid with *o*-phenylenediamine[7,8] was utilized for pre-column derivatization to determine dehydroascorbic acid and isodehydroascorbic acid in foodstuffs by a high-performance liquid chromatography (HPLC) method[9].

We have recently found that pyrimidine bases that have a six-membered ring with a conjugated double bond could be separated from each other by isocratic

0021-9673/85/$03.30

elution from a column of a hydrophilic gel, Asahipak GS-320, using an aqueous buffer as the eluent[10]. As ascorbic acid has a five-membered ring with a conjugated double bond, we tried to separate ascorbic acid from dehydroascorbic acid and diketogulonic acid, and a good separation was obtained at pH 3.0 using dilute tartrate buffer containing ethylenediaminetetraacetate (EDTA) and β-thiodiglycol as the mobile phase. A fluorimetric method using benzamidine as the post-column derivatizing agent[11] allowed the simultaneous determination of ascorbic acid, dehydroascorbic acid, diketogulonic acid and glucose.

EXPERIMENTAL

Materials and solutions

All chemicals were of analytical-reagent grade (Yashima Pharmaceutical, Osaka, Japan) and water was of ultrapure grade prepared by reverse osmosis (RO-pure 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 EDTA and 1 g of β-thiodiglycol in 2 l of ultrapure water and adjusting the pH to 3.0 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 primary stock solution of ascorbic acid was prepared by dissolving 20 mg of ascorbic acid in 100 ml of mobile phase. Secondary standards were prepared by diluting the stock solution with the mobile phase to give solutions containing 1, 2, 4, 10 or 20 mg/l of ascorbic acid. These standard solutions were prepared daily.

Dehydroascorbic acid was prepared by oxidation of ascorbic acid with bromine water. Ten milligrams of ascorbic acid were dissolved in 10 ml of water and bromine water was added drop by drop until a slight yellow colour remained. Excess of bromine was destroyed by addition of three drops of a 2% (w/v) solution of β-thiodiglycol and the pH of the mixture was adjusted to 2.5 with 0.5 *M* sodium hydroxide solution. The mixture was diluted with water to 50 ml and an aliquot of the solution was diluted with mobile phase to give a solution of 4 mg/l. This was analysed immediately.

Diketogulonic acid was prepared by hydrolysis of dehydroascorbic acid.

The solution of oxidized ascorbic acid described above was neutralized to pH 7 with 0.5 *M* sodium hydroxide at 30°C. The solution was then diluted to 50 ml with water and an aliquot of the solution was diluted with mobile phase to give a solution of 2 mg/l.

Reagent A was a solution of benzamidine hydrochloride (0.02 *M*) and reagent B was potassium borate buffer of pH 10 (0.25 *M*) containing 0.25 *M* potassium sulphite.

Equipment

The chromatography system consisted of a constant delivery pump (Trirotar III; Jasco, Japan), an automatic injector (Model KSST 60J; Kyowa Seimitsu, Japan) and a column of hydrophilic gel (Asahipak GS-320, 50 × 0.76 cm I.D.; Asahi Chemical, Japan). A dual-head pump (Model SP-024-2; Jasco) was used to pump the reagents and mix them with the eluate. A Model FP-210 spectrophotometer (Jasco, Japan) equipped with a flow cell (volume 15 μl) was used to monitor fluorescence.

Preparation of mixed bed column

Amberlite CG-50 was graded according to size, washed as described previously[12] and converted into the hydrogen ion form. Amberlite XAD-2 was washed with methanol on a glass filter until the absorbance of the effluent became 0.2 and then washed with water. Equal volumes of Amberlite CG-50 and XAD-2 were mixed, washed with mobile phase and stored in it. The mixed resin was poured into a chromatographic tube (10 × 0.6 cm I.D. with a 5-ml reservoir) and allowed to settle under gravity to form a resin bed 2 cm high.

Purification of fruit juice

A 1-ml volume of orange juice was placed on a mixed-bed column and, after the sample had passed into the column, it was washed with 4 ml of mobile phase. The effluent was collected in a 50-ml measuring flask and diluted with mobile phase to 50 ml. A volume of 10 μl of the sample was used for analysis.

Chromatographic separation and fluorimetric determination

A 10–50-μl volume 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 0.4 ml/min each and mixed using a T-shaped connector. The mixture was heated at 90°C in a PTFE tube (length 30 m, I.D. 0.5 mm, O.D. 1.5 mm) 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, dehydroascorbic acid and ascorbic acid were eluted in that order within 30 min. Optimum of the four com-

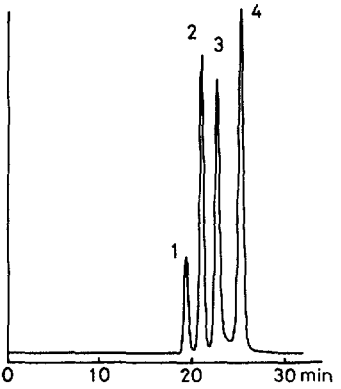

Fig. 1. Elution pattern of glucose, diketogulonic acid, dehydroascorbic acid and ascorbic acid. Peaks: 1 = glucose (3000 ng); 2 = diketogulonic acid (33 ng); 3 = dehydroascorbic acid (30 ng); 4 = ascorbic acid (120 ng). As 1–2% of dehydroascorbic acid and diketogulonic acid were detected in freshly prepared samples of ascorbic acid and dehydroascorbic acid, respectively, the heights of peaks 2 and 3 will be greater than those given by pure samples.

pounds was achieved at pH 3.0. The elution volumes of diketogulonic acid and ascorbic acid were sensitive to changes in the pH of the mobile phase, but those of glucose and dehydroascorbic acid were not. Therefore, at lower pH, the separation of glucose from diketogulonic acid and ascorbic acid from dehydroascorbic acid was improved, whereas diketogulonic acid and dehydroascorbic acid were eluted closer together. At higher pH, the reverse was observed. The optimum pH for the reaction of ascorbic acid with benzamidine to form fluorescent compound(s) was 9.1–9.2. The peak height of ascorbic acid was reproducible. When 40 ng of ascorbic acid were injected, the mean peak height was 83.5 ± 1.2 mm (S.D.) (six determinations), and a linear relationship between peak height and amount of ascorbic acid injected was observed over the range 5–500 ng.

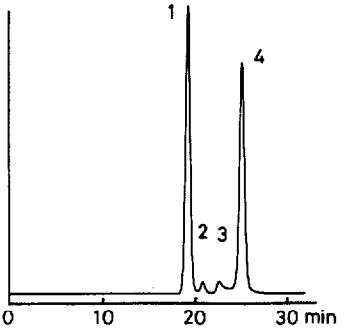

Fig. 2. Elution pattern of an extract of orange juice. Volume of extract injected, 10 μl. Peaks as in Fig. 1.

The elution pattern of the orange juice extract is shown in Fig. 2. The elution volume of the last peak corresponds to that of ascorbic acid, and from the peak height of the last peak the amount of ascorbic acid in the orange juice was calculated to be 40 mg per 100 ml. As ascorbic acid is eluted isocratically from Asahipak GS-320, regeneration of the column is not necessary. Therefore, this method will be useful for the routine determination of ascorbic acid in juice samples. The application of this method to the determination of ascorbic acid, dehydroascorbic acid and diketogulonic acid in other biological samples will be reported later.

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