Journal of Chromatography, 633 (1993) 245–250
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 756

Measurement of vitamin C by capillary electrophoresis in biological fluids and fruit beverages using a stereoisomer as an internal standard

Edward V. Koh, Michael G. Bissell and Ralph K. Ito*

Nichols Institute, Nutrition and Metabolism Biochemistry Department, 33608 Ortega Highway, San Juan Capistrano, CA 92690-6130 (USA)

(First received June 11th, 1992; revised manuscript received November 13th, 1992)

ABSTRACT

Ascorbic acid (or vitamin C) is an important component of many biological systems and various physiological roles have been described for it. A rapid and simple capillary electrophoresis method for ascorbic acid measurements in biological fluids as well as in beverages was developed. A stereoisomer of ascorbic acid, isoascorbic acid, not normally found in nature, was used as the internal standard for this assay. The analysis was performed in a 30 cm × 75 μm I.D. fused-silica capillary with 100 m*M* tricine buffer, pH 8.8, and measured by UV absorbance at 254 nm. The method was sensitive to 1.6 μg/ml and linear to 480.1 μg/ml. Within-run R.S.D. was 3.2% (93.5 ± 3.0 μg/ml, mean ± S.D., n = 18) and run-to-run R.S.D. was 3.3% (35.6 ± 1.2 μg/ml, mean ± S.D., n = 10) and 1.9% (149.4 ± 2.8 μg/ml, mean ± S.D., n = 10). Average spiked recovery from human plasma samples was 98.0%. The technique has been demonstrated to be suitable for assay of vitamin C in biological samples and some fruit juices.

INTRODUCTION

Ascorbic acid is the most abundant biologically active form of vitamin C in humans. Analysis of ascorbic acid by present techniques is both time consuming and complicated [1–4]. High-performance liquid chromatography (HPLC) methods have been used for vitamin C analysis. Examples are cation-exchange chromatography [5], ion-exclusion chromatography [6], and reversed-phase chromatography [7–13]. Some minor problems with ascorbic acid measurement have been observed when using amperometric detectors [6] and ultraviolet detectors [14]. These problems have been related to working electrode contamination of amperometric detectors and ultraviolet interfering components in biologic fluids.

Capillary electrophoresis (CE) is a useful analytical separation technique known for its high resolution and small sample volume. The number of CE applications is rapidly growing and includes some interesting applications in the clinical field [15]. The analysis of urinary components by CE also has been reported [16–18]. More recently, several papers have demonstrated the usefulness of micellar electrokinetic capillary chromatography (MECC) for separation of various groups of compounds, including water and fat-soluble vitamins [19]. These reports on vitamins were focused on the separation techniques and did not reach low detection limits of 1.6 μl/ml for clinical applications where <2.0 μg/ml is considered deficient [3].

The main objective of this investigation was to develop a simple and rapid method for vitamin C analysis in biological fluids using a commercially available CE apparatus. By developing a CE method using isoascorbic acid as an internal standard, a fully automated quantitative analysis of vitamin C in plasma, serum, urine and some beverages has been developed.

* Corresponding author.

0021-9673/93/$06.00

EXPERIMENTAL

Instrumentation

A Model 2100 high-performance capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) was used for CE studies. A personal integrator system (Model 1020; Perkin-Elmer, Norwalk, CT, USA) was used for data handling. A refrigerated centrifuge (Model J-6B; Beckman Instruments, Palo Alto, CA, USA), and a multitube vortexer (VWR Scientific, Philadelphia, PA, USA) were used for sample extractions.

The capillary (Polymicro Technologies, Scottsdale, AZ, USA) size was 37 cm × 75 μm I.D. and length to the detector was 30 cm. The polyimide coating at the detector window was removed by flaming followed by methanol wash. After installing the capillary column into a capillary cartridge, the capillary was conditioned by pressuring with 1 *M* sodium hydroxide for 10 min, water for 10 min, and the run buffer for 10 min.

Reagents

The following reagents were purchased from Sigma (St. Louis, MO, USA): EDTA, trichloroacetic acid, L-ascorbic acid; D-isoascorbic acid; uric acid standard solution and tricine. Sodium hydroxide was purchased from Fisher Scientific (Fair Lawn, NJ, USA), Ascorbic acid oxidase from Boehringer Mannheim (Indianapolis, IN, USA), and metaphosphoric acid from Aldrich (Milwaukee, WI, USA). Reagent-grade deionized water from EM Science (Gibbstown, NJ, USA) was used to prepare all buffers. Biocell plasma (Biocell Labs., Rancho Dominguez, CA, USA) lots were screened and those deficient in ascorbic acid were used to make plasma standard and controls.

Procedures

Fresh urine and whole blood were collected and treated to obtain plasma (EDTA, 0.1 m*M*) or serum (after clotting) from volunteers. Volunteers were asked to fast overnight prior to sample collection. All the urine samples were preserved by adding metaphosphoric acid (MPA, 100 g/l) and then stored at −80°C. Samples were stored up to 4 weeks and were run in batches. Urine and beverage samples for the analysis were prepared by diluting 0.2 ml of sample with 0.1 ml of MPA (100 g/l) in a 75 × 12 mm test tube which also contained 0.1 ml isoascorbic acid (100 μg/ml) as an internal standard. Samples were vortex-mixed for 15 s and filtered through a 0.45-μm filter by centrifugation (3000 *g*, 10 min, 5°C). The filtrate was transferred to a Beckman CE microvial and analyzed. Aliquots of plasma or serum (0.5 ml) were mixed with 0.5 ml of 12% trichloroacetic acid (TCA) to deproteinate sample and extract analytes into the aqueous phase. The TCA also preserves the ascorbic acid. The solution was vortexed for 30 s. After centrifugation for 5 min at 5°C, the supernatants (0.7 ml) were filtered and 100 μl of isoascorbic acid (81.5 μg/ml) were added to 0.4 ml of filtrate for analysis.

Two lots of urine and plasma controls were prepared by supplementation of known amounts of ascorbic acid such that the final concentration of ascorbic acid in each lot was 35.6 and 149.4 μl/ml of urine, and 3.5 and 20.0 μg/ml of plasma (low end and high end of expected adult reference range, respectively).

Samples were injected into the separation capillary by the pressure injection method. Separation voltage was set at 11 kV (297 V/cm) across the capillary and on-column UV absorption at 254 nm was used for measurement. After each separation, the capillary was rinsed sequentially with 0.1 *M* NaOH, distilled water, and buffer, for 1 min each, between successive electrophoretic runs.

RESULTS AND DISCUSSION

Mobility (migration time) can change during CE separation and this is noted to occur from run-to-run [20]. It is important to minimize migration time variation in the sample analysis for proper peak identification. Sample pretreatment and extraction methods are often used to resolve sample-to-sample variation and retention time drift problems with HPLC [5,6,7–13]. This can be done by traditional methods such as solid-phase or liquid–liquid phase extractions then followed by sample analysis. Plasma samples for CE analysis were processed using TCA as the liquid–liquid extraction method which also served as a preservative for ascorbic acid. The samples became homogenous by extraction and the procedure reduced the sample's matrix effects on migration time and improved means of peak identification by reducing interference. For CE analysis

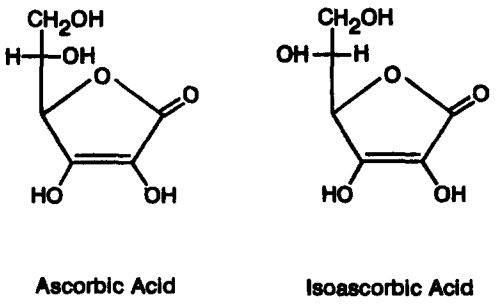

Fig. 1. Structure of ascorbic acid and one of its stereoisomers, isoascorbic acid.

in urine and beverage samples, concentrated metaphosphoric acid (100 g/l) was added as a preservative for ascorbic acid. Additional sample pretreatment was not found to be necessary. All the analytical results after these sample pretreatment procedures showed significant improvement in sample-to-sample variation and reduced retention time drift.

It is most desirable to use a suitable internal standard for quantitative analysis when possible. Ascorbic acid is the biologically important analyte to measure [21]. One of its stereoisomers, isoascorbic acid, which does not naturally exist in humans or

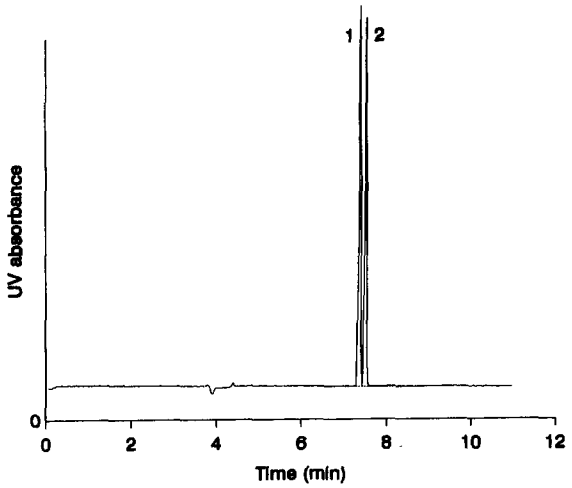

Fig. 2. Electropherogram of ascorbic acid (a) and isoascorbic acid (2) using 100 μg/ml each in metaphosphoric acid solution (100 g/l). Procedure conditions: buffer, 100 m*M* tricine (pH 8.8); capillary, uncoated 37 cm × 75 μm I.D. (30 cm to detector); applied voltage, 11 kV.

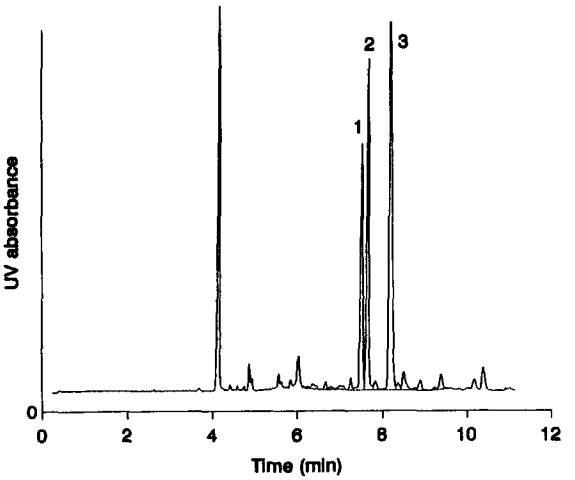

Fig. 3. Electropherogram of a human urine. Peaks: 1 = ascorbic acid (17.5 μg/ml); 2 = isoascorbic acid (85.0 μg/ml); 3 = uric acid. CE separation conditions as in Fig. 2.

other natural products, was selected as an internal standard. Manufacturers of some foods or beverages may add isoascorbic acid to products as an additive. The addition of isoascorbic acid as an internal standard for ascorbic acid analysis in foods or beverages which may contain isoascorbic acid as an antioxidant will result in erroneous values for ascorbic acid. In this case, the native level of isoascorbic acid must be established by testing the sample prior to addition of the isoascorbic acid. Ascorbic acid and isoascorbic acid differ from each other only in the way the atoms are oriented in space (Fig. 1). These physical properties of stereoisomers make them difficult to distinguish by usual analytical methods. Several interesting MECC techniques have been reported which include the separation of stereoisomers [22].

The method we report here is a novel CE procedure (not MECC), which separates these two stereoisomers with baseline resolution as shown in Fig. 2. It is favorable to have an internal standard migrating near the analyte peak for CE applications. The use of a stereoisomer as an internal standard is highly desirable as conditions that effect the analyte peak, similarly effect the internal standard peak. We have observed that the effect of changing electrophoretic conditions (*i.e.*, pH, ionic strength) has little influence on quantifying or identifying the ascorbic acid peak [22]. Some useful assay applications

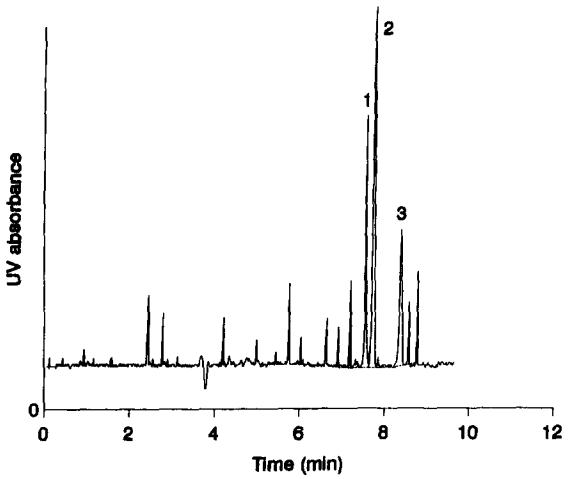

Fig. 4. Electropherogram of a human plasma which was extracted with 12% trichloroacetic acid. Peaks: 1 = ascorbic acid (5.8 μg/ml); 2 = isoascorbic acid (16.3 μg/ml); 3 = uric acid. CE separation conditions as in Fig. 2.

are shown for ascorbic acid measurements in urine (Fig. 3), plasma (Fig. 4), and fruit juice (Fig. 5). In addition, the uric acid peak (Figs. 3 and 4) which was consistently observed in urine and plasma was identified using a uric acid standard solution by standard recovery studies and retention times. These applications show that CE is a useful analytical method for the quantitative analysis of ascorbic

TABLE I

REPRODUCIBILITY OF ASCORBIC ACID IN URINE FOR BOTH INTRA-RUN AND INTER-RUN

	n	Mean ± S.D. (μg/ml)		R.S.D. (%)
Intra-run	18	93.5	3.0	3.2
Inter-run				
Level 1	10	35.6	1.2	3.3
Level 2	10	149.4	2.8	1.9

acid and the selection of rare isomers can be ideal internal standards for analysis.

Metaphosphoric acid was previously reported to be used as preservative for ascorbic acid [5]. We have found that both urine samples can be preserved with MPA and plasma with TCA without interfering with the CE method. These preservatives maintained reproducibility of ascorbic acid in both intra-run and inter-run analysis (Table I). As shown in Table I, excellent relative standard deviations were recorded from run-to-run (*i.e.*, intra-run, R.S.D. = 3.2%) and day-to-day (*i.e.*, inter-run, level 1, R.S.D. = 3.3% and level 2, R.S.D. = 1.9%).

We examined interference due to the biological sample matrix by standard recovery studies. A known amount of ascorbic acid standard (34.5 μg/ml) was spiked into serum samples (n = 5). Recovery of the added standard averaged 98% which shows excellent analytical accuracy in a complex

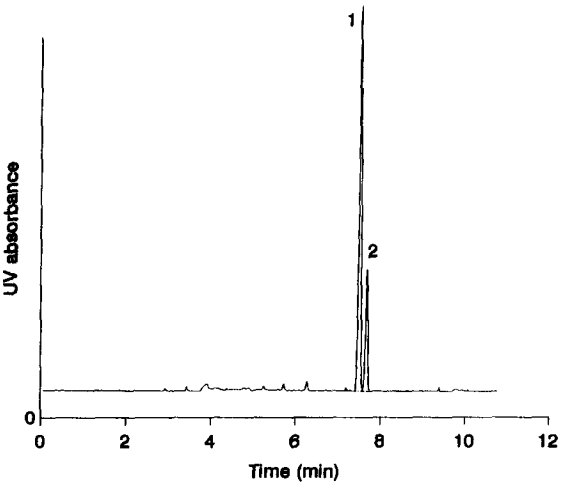

Fig. 5. Electropherogram of a name brand juice. Peaks: 1 = ascorbic (329.9 μg/ml); 2 = isoascorbic acid (100.0 μg/ml). CE separation conditions as in Fig. 2.

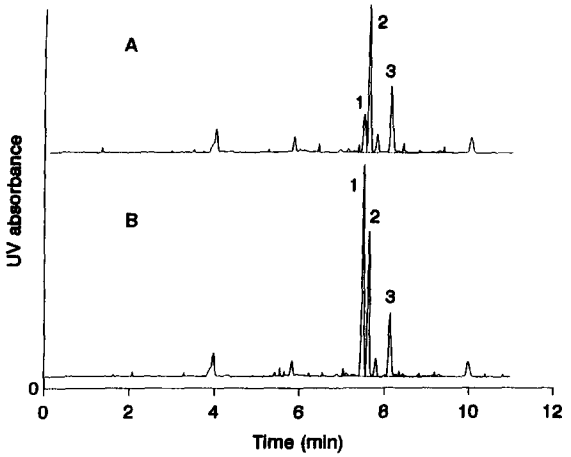

Fig. 6. Electropherogram of human urine (A) and spiked human urine (B). Peaks: 1 = ascorbic acid; 2 = isoascorbic acid; 3 = uric acid. CE separation conditions as in Fig. 2.

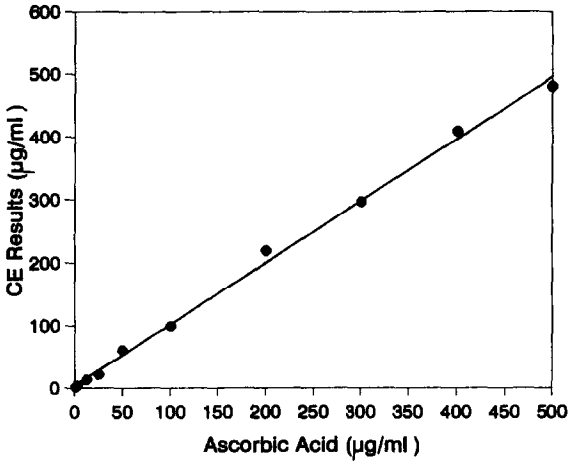

Fig. 7. Linearity curve obtained with a CE assay and a known ascorbic acid standard. The linear regression equation found was $y = 1.0149x - 4.2632$, $r = 0.9984$.

matrix. Similar results were obtained in urine matrix. The spiked electropherogram is shown in Fig. 6. In addition, we further demonstrate analytical specificity with use of ascorbate oxidase to remove ascorbic acid from sample solution. We added 17 U ascorbic acid oxidase to 3.0 ml of urine and incubated at 23°C for 6.0 min. This results in a smaller ascorbic acid peak as the ascorbic acid in the sample solution is oxidized to dehydroascorbic acid.

Linearity and sensitivity of the assay is shown in Fig. 7 using an aqueous standard. The method was sensitive to 1.6 µg/ml (lowest measurable level above background) and linear to 480.0 µg/ml. The linear regression and correlation equation with known ascorbic acid standard samples was $y = 1.0149x - 4.2632$, $r = 0.9984$. Satisfactory signal-to-noise ratios were observed at sensitive levels needed to cover the vitamin C deficiency cases in plasma or serum (less than 2.0 µg/ml) [3]. In addition, those patients that supplemented themselves with large oral dosages of vitamin C also can be evaluated.

We tested the application of this assay to measure vitamin C in several different types of fruit juices and a wine sample. The measurements were accomplished with use of the same electrophoretic system as described previously for the analysis of vitamin C in plasma and urine. The concentration of vitamin C in these beverages is shown in Table II. It is of interest to note that a considerable difference of vitamin C concentration was found between beverages.

TABLE II

ASCORBIC ACID DETERMINATION IN BEVERAGES

Sample	Ascorbic acid (µ/ml)
Orange juice	329.9
Apple juice	356.1
Grapefruit juice	103.8
Vegetable juice	344.4
Cranberry cocktail	>1.6
White wine	>1.6

The open-tube CE system is an excellent and powerful analytical system when considering that only a simple buffer solution was used to separate ascorbic acid from its stereoisomer, isoascorbic acid. It is applicable to a large variety of biological samples, many of which have been accomplished with HPLC [5–13,23,24] but are more labor intensive. This application of CE demonstrates excellent separation characteristics between ascorbic acid and isoascorbic acid and appears to be an improvement over HPLC means of stereoisomer separation and analysis. From the data we presented, this separation technique is well suited for clinical evaluation of human vitamin C status. Vitamin C has been reported to be an important antioxidant and nutrient which has been shown to increase longevity 6 years in men and 2 years in women [25]. The simplicity of this method suggests that vitamin C levels and distribution (ingestion, absorption and excretion) can now be easily monitored in body fluids.

REFERENCES

1 M. R. H. Lowik, J. Schrijver and M. Wedel, *Int. J. Vit. Nutr.*, 61 (1991) 43.
2 J. M. Kim, K. Huang and R. D. Schmid, *Anal. Lett.*, 23 (1990) 2273.
3 N. W. Tietz, *Clinical Guide to Laboratory Tests*, Saunders, Philadelphia, PA, 1990.
4 A. J. Pesce and L. A. Kaplan, *Methods in Clinical Chemistry*, Mosby, St. Louis, MO, 1987.
5 S. A. Margolis and T. P. Davis, *Clin. Chem.*, 34 (1988) 2217.
6 H. P. Wagner and M. J. McGarrity, *J. Chromatogr.*, 546 (1991) 119.
7 M. A. Kutnink, J. H. Skala, H. E. Sauberlich and S. T. Omaye, *J. Liq. Chromatogr.*, 8 (1985) 31.

8 M. C. Gennaro and C. Abrigo, *Fresenius J. Anal. Chem.*, 340 (1991) 422.
9 J. Cammack, A. Oke and R. H. Adams, *J. Chromatogr.*, 565 (1991) 529.
10 N. Bilic, *J. Chromatogr.*, 543 (1991) 367.
11 G. B. de Quinga, M. Lopez-Torres, R. Perez Campo and C. Rojas, *Anal. Biochem.*, 199 (1991) 81.
12 K. P. Dhariwal, W. O. Hartizell and M. Levine, *Am. J. Clin. Nutr.*, 54 (1991) 712.
13 C. S. Tsau and S. D. Salimi, *J. Chromatogr.*, 245 (1982) 355.
14 G. Lazzarino, D. D. Pierro, B. Tarazzi, L. Gerroni and B. Giardina, *Anal. Biochem.*, 197 (1991) 191.
15 W. G. Kuhr, *Anal. Chem.*, 62 (1990) 403R.
16 N. A. Guzman, M. A. Trebelcock and J. P. Adris, *J. Liq. Chromatogr.*, 14 (1991) 997.
17 I. Z. Atamna, G. M. Janini, G. H. Muschik and H. J. Issaq, *J. Liq. Chromatogr.*, 14 (1991) 427.
18 W. Thormann, P. Meier, C. Marcolli and F. Binder, *J. Chromatogr.*, 545 (1991) 445.
19 C. P. Ong., C. L. Ng., H. K. Lee and S. F. Y. Li, *J. Chromatogr.*, 547 (1991) 419.
20 T. Tsuda, K. Nomura and G. Nakagawa, *J. Chromatogr.*, 264 (1983) 385.
21 N. W. Tietz, *Fundamentals of Clinical Chemistry*, Saunders, Philadelphia, PA, 1987.
22 S. Terabe, M. Shibata and Y. Miyashita, *J. Chromatogr.*, 480 (1989) 403.
23 M. C. Gennaro, C. Abrigo and E. Marengo, *Chromatographia*, 30 (1990) 311.
24 S. A. Margolis, R. C. Paule and R. G. Ziegler, *Clin. Chem.*, 36 (1990) 1750.
25 G. Cowley and V. Church, *Newsweek*, May 18 (1992) 60.