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Note

High-performance liquid chromatographic determination of ascorbic acid in urine

Effect on urinary excretion profiles after oral and intravenous administration of vitamin C

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Methods for analyzing the ascorbic acid content of biological fluids such as urine have traditionally been titrimetric [1] and/or spectrophotometric [2]. These techniques, although quite accurate, are laborious and time-consuming. More recently, high-performance liquid chromatography (HPLC) has been applied to the measurement of ascorbic acid in foods, multi-vitamin products, and biological fluids [3, 4]. We have used HPLC to determine the ascorbic acid content of urine samples, which is more accurate than the traditional methods of analysis and which enables one to obtain a direct readout of the amount of ascorbic acid in a sample within minutes. In the course of this investigation, urinary excretion profiles for orally ingested and intravenously infused doses of ascorbic acid have been determined and compared to examine the role the absorptive process plays on the reported [5] diminished urinary excretion of ascorbic acid over the 500-mg dosage.

EXPERIMENTAL

Materials

Ascorbic acid (1.0-g tablets), commercially obtained, were used by the nine subjects for the oral ingestion portion and a 10-ml ampoule of Cevalin, 1 g (Eli Lilly & Co., Indianapolis, Ind., U.S.A.), was used for the intravenous infusion portion of this experiment.

A standard ascorbic acid solution was prepared using 100 mg ascorbic acid

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(Fisher Scientific, Pittsburgh, Pa., U.S.A.) per liter 10% metaphosphoric acid (Fisher Scientific). The titration dye solution was prepared using 80 mg 2,6-dichlorophenol-indophenol (Sigma, St. Louis, Mo., U.S.A.) per 500 ml of solution containing 12 ml of phosphate buffer at pH 7.0.

The mobile phase of the HPLC analysis was 0.8% metaphosphoric acid prepared by adding 8 g metaphosphoric acid (Fisher Scientific) to double-distilled, Millipore-filtered water (1000 ml). This solution was de-aerated by vacuum.

Sample collection

Nine healthy subjects ingested 1.0 g of ascorbic acid for 13 days. On the 7th day each subject collected urine samples at each of the following designated times: before ingestion of the tablet and 3, 6, 9, 12, 15 and 24 h after ingestion of the tablet. On the 14th day each subject received a 1.0-g intravenous infusion of the vitamin and again collected urine samples at the times designated above. The volume of urine collected at each time interval was recorded, and the sample submitted for analysis was acidified with 10% metaphosphoric acid (5 volumes of urine plus 1 volume of acid).

Titrimetric procedure

Ascorbic acid present in the urine samples was determined by titration with the dye, 2,6-dichlorophenol-indophenol, solution. This method depends on the reducing power of ascorbic acid. Since other reducing substances may interfere with the determination of ascorbic acid, the titration was performed rapidly in acid solution. A 10-ml aliquot of the dye solution is titrated by the constant dropwise addition of the sample of urine which has been acidified (the dye solution is blue at the start but changes to deep red upon addition of the first few drops of acidified urine). The end point is indicated by the disappearance of the red color. All determinations were made in triplicate.

High-performance liquid chromatography

Apparatus and operating conditions. A Model ALC-202 liquid chromatograph equipped with a Model 6000 solvent delivery system (Waters Assoc., Milford, Mass., U.S.A.) was used in the study. The column effluents were monitored with the 254-nm detector. Samples were introduced into the system via syringe injection. The flow-rate was 3.0 ml/min.

Column. A 30 cm × 4 mm I.D. μBondapak C_{18} column (Waters Assoc.) was used. μBondapak has a monomolecular layer of octadecyltrichlorosilane chemically bonded to μPorasil beads having an average particle size of 10 μm. The number of theoretical plates, based upon ascorbic acid at 3.0 ml/min was 2300. The chromatographic system was flushed nightly with methanol, as recommend by the supplier, ensuring removal of lipophilic substances from the column.

Standard curve. Samples of ascorbic acid in 0.8% metaphosphoric acid were prepared to contain 0.50, 0.75, 1.00, and 1.50 mg of ascorbic acid per ml. Aliquots (5.0 μl) of these solutions were injected into the chromatographic system, and the resulting peak heights were plotted against concentration

for the calibration curve. Each point was the average of twelve injections. The reliability to the straight line was 0.993, the slope was 0.3448 and the y intercept was −0.0012 integrator units. These data indicate the procedure is amenable to use of a single-point standard.

Mobile phase. Metaphosphoric acid (0.8%) prepared with double-distilled, Millipore-filtered water was de-aerated by vacuum before use.

Sample preparation. Each urine sample was filtered with a sample clarification kit (Waters Assoc.) which can remove fine particles of 0.5 μm or greater. A 5-μl portion of the acidified urine sample was injected, the resulting peak height determined, and the quantity of ascorbic acid in the sample calculated by reference to the previously derived calibration curve. Knowing the total volume of urine excreted at each time allowed the calculation of the total amount of ascorbic acid at the time in question. All determinations were made in triplicate.

RESULTS AND DISCUSSION

Results of the determination of ascorbic acid in urine using a modification of the chromatographic procedure described by Sood et al. [3] were compared (Table I) with results of the 2,6-dichlorophenol-indophenol titration. The titration procedure was used for comparison because of the wide use of the method. The precision of the visual titration procedure is considerably reduced because of the subjectiveness of end point determinations.

The ideal mobile phase to determine ascorbic acid in urine is 0.8% metaphosphoric acid solution. Other mobile phases [including ammonium salts in methanol—water (50:50)] resulted in values for ascorbic acid that were too high by 6—12%. This was attributed to the presence of other substances

TABLE I

COMPARISON OF URINARY ASCORBIC ACID LEVELS OBTAINED BY THE TWO METHODS

Urine sample No.*	Concentration of ascorbic acid (mg/ml) ± S.D.		
	Amount of ascorbic acid added to urine sample	Titrimetric method**	HPLC method**
1	2.0	1.1 ± 0.1	2.6 ± 0.1
2	20.0	15.2 ± 0.9	20.6 ± 0.1
3	35.0	34.2 ± 1.7	35.1 ± 0.2
4	62.5	61.1 ± 1.8	64.7 ± 0.2
5	62.5	62.8 ± 1.2	65.7 ± 0.5
6	20.0	20.3 ± 1.3	20.0 ± 0.1
7	10.0	9.8 ± 0.5	10.6 ± 0.1

*Urine sample spiked with ascorbic acid.
**Average of triplicates.

in urine (e.g., uric acid) which exhibit peaks corresponding to the retention volume of ascorbic acid. A representative chromatogram of ascorbic acid in urine using the 0.8% metaphosphoric acid solution mobile phase is presented in Fig. 1. A problem which was encountered with other mobile phases but not with 0.8% metaphosphoric acid solution was that when the chromatographic column had not been used for some time (ca. 45—60 min), the first sample injected always gave a value that was 7—14% low. Once the column had been pretreated with the first sample, quantitation of subsequent injections was precise. The use of 0.8% metaphosphoric acid solution as the mobile phase eliminated this phenomenon, with the result that the quantitation of all injections was accurate and precise no matter what the time interval between injections.

This liquid chromatographic method was used to determine the urinary excretion profiles for both orally ingested and intravenously infused 1.0-g doses of ascorbic acid. A plot of the mean urine concentrations of ascorbic acid after the oral dose and the intravenous infusion of ascorbic acid with time in 9 subjects (Fig. 2) showed that the peak of mean concentrations occurred at 3 h and 6 h for the infused and oral doses, respectively.

The data show that the urinary excretion of the orally ingested 1000-mg doses of vitamin C (610±65 mg) using 9 subjects is in agreement with our earlier study [5]. The results of this earlier study involving 24 healthy subjects receiving daily oral doses of 100, 250, and 500 mg ascorbic acid indicated that they excreted in their urine amounts of ascorbic acid equal to the amount

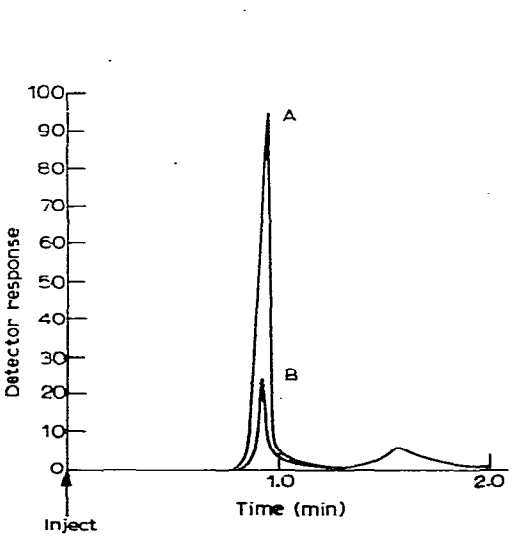

Fig. 1. HPLC chromatogram of: (A) 1.78 mg/ml ascorbic acid in urine; (B) 1:4 dilution of (A). Flow-rate, 3.0 ml/min; chart speed 2 in./min; range = 64.

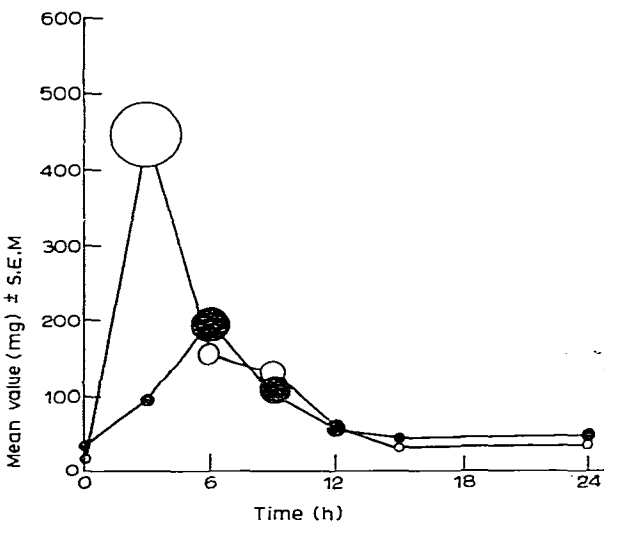

Fig. 2. Mean±S.E.M. values of ascorbic acid excreted by 9 subjects in 24 h versus time after ingestion and infusion of 1.0 g of the vitamin. •, Oral ingestion; 610±65 mg excreted. ○, Intravenous infusion; 930±60 mg excreted.

ingested. With doses of 1.0 and 2.0 g per day, urinary excretion dropped to 54% and 36% of the administered dose, respectively. In addition, a dramatic increase in the urinary excretion of ascorbic acid (930±60 mg) is demonstrated when these same 9 subjects receive the vitamin via intravenous infusion. Consequently, if the route of administration of the vitamin is intravenous infusion rather than oral ingestion (by this means circumventing the intestinal absorption of the vitamin), over 90% of the infused dose is excreted in the urine. Therefore, large portions of single oral doses of ascorbic acid greater than 500 mg cannot be accounted for by urinary excretion because there is a decrease in the efficiency of ascorbic acid intestinal absorption at the higher dosage levels.

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