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Short Communication

Measurement of ascorbic acid in human plasma and urine by high-performance liquid chromatography Results in healthy subjects and patients with idiopathic calcium urolithiasis

M. Manoharan, P.O. Schwille *

Mineral Metabolism and Endocrine Research Laboratory, Departments of Surgery and Urology, University Hospital, Postfach 3560, D-91023 Erlangen, Germany

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Abstract

A simple, reliable high-performance liquid chromatographic method was developed to measure ascorbic acid (ASC), with ultraviolet detection (250 nm), in human plasma and urine. Immediately following blood withdrawal, the heparinized plasma samples were deproteinized with 10% *m*-phosphoric acid, while the freshly voided urine samples were diluted with *m*-phosphoric acid. ASC was separated on a reversed-phase column by elution with 0.1 *M* KH_2PO_4 adjusted to pH 2.35. In urine, after reduction of dehydroascorbic acid to ASC, total ASC was measured using the same mobile phase. The method was sensitive down to 0.1 and 0.4 mg ASC per litre of urine and plasma, respectively. In patients with idiopathic calcium urolithiasis, both plasma and urinary ASC were within the range observed in age-matched controls.

1. Introduction

In the past, numerous methods for analyzing ascorbic acid (ASC) in biological fluids have been proposed (for review see Pachla *et al.* [1]. Most of the procedures are either time-consuming, due to the need for sample pretreatment, or are not specific and not sensitive enough; in addition, they require electrochemical or fluorimetric detection, techniques not generally available in hospital laboratories. Only a few recently described methods [2,3], can be applied by clinical laboratories equipped with high-performance liquid chromatography systems without the need for ion-pairing reagents or organic modifier. The studies mentioned [2,3] separated plasma ASC on a C_{18} column. Hallstrom *et al.* [4] used, as did we in this study, an eluent at pH 2.4, but for deproteinization still needed to dialyse samples – as described by Harapanhalli *et al.* [3].

From a critical examination of existing procedures we concluded that a method employing an eluent similar to that described by Hallstrom *et al.* [4], in combination with a higher capacity column (C_{30}), should result in superior separation. If this is true, separation at pH 2.3 would

* Corresponding author.

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permit simple sample processing. Low ASC concentration in the plasma of healthy humans, around 2.5–3.0 mg/l, are well above the sensitivity of the method described in the present study. Also in the present work, urine ASC was separated on a C_{18} column, but with an initially low flow-rate, until detection of ASC.

In fresh plasma, the dehydroascorbic acid concentration is almost constant, and is less than 5% of total ASC [5,6]. Conversely, findings in our laboratory show that dehydroascorbic acid in freshly voided urine varies between 5 and 15% of total ASC. Dehydroascorbic acid does not absorb ultraviolet light at the same wavelength as ASC, thus requiring a reduction in the urinary dehydroascorbic acid with dithiothreitol before injection for measurement of total ASC, a procedure analogous to that used in other laboratories.

In addition, we evaluated the state of ASC in urine and plasma of calcium urolithiasis patients, and present preliminary data. An understanding of the pathophysiology of this common clinical disorder necessitates a thorough knowledge of the intake and metabolism of ASC, which is an oxalate precursor both *in vitro* [7] and *in vivo* [8–10].

2. Experimental

2.1. Instrumentation

The HPLC system consisted of the Jasco 880-PU Intelligent pump (ESWE, Sinsheim, Germany); Micromeritics Autosampler (Bischoff, Leonberg, Germany); electrically operated Rheodyne valve with a 10-μl sample loop; Triacontyl = Daltosil 100 column (4 μm), 250 × 4 mm I.D. (Serva, Heidelberg, Germany); Ultrasphere column (5 μm), 250 × 4 mm I.D. (Beckman, Munich, Germany); Jasco 870-UV Spectralphotometer (ESWE); Apex computer integration system (ESWE).

2.2. Chemicals

Only analytical grade substances were used: ascorbic acid, potassium dihydrogen phosphate, dithiothreitol, *o*-phosphoric acid and *m*-phosphoric acid (all from Merck, Darmstadt, Germany); Ascorbate oxidase spatula (Boehringer, Mannheim, Germany). ASC stock solution was prepared by dissolving 20 mg of ASC in 100 ml 2.5% *m*-phosphoric acid, and aliquots were stored at −80°C. For measuring ASC in plasma, standard solutions were prepared by diluting 25, 50, 75 and 100 μl of the stock solution in water and making up to a volume of 1 ml; for urine ASC, the stock solution served as the standard solution (200 mg/l).

2.3. Sample storage and pretreatment

Blood was drawn into a heparinized syringe from a forearm vein, transferred to prechilled tubes, centrifuged at 4°C for 3 min at 8000 *g*, and the plasma deproteinized with 10% *m*-phosphoric acid (3:1, v/v); the clear supernatant was snap frozen in liquid nitrogen, and then stored at −80°C. After thawing, this sample was immediately injected onto the HPLC system for ASC measurement. Freshly voided fasting urine was immediately passed through a filter paper (Schleicher–Schüll, Dassel Germany), snap frozen in liquid nitrogen, and stored at −80°C until analysis. To measure ASC, urine was thawed, diluted 6 times with 0.05% *m*-phosphoric acid and then injected. Another urine aliquot was diluted with an equal volume of dithiothreitol (200 mmol/l) and allowed to stand at room temperature for 30 min. Thereafter, this solution was diluted 3 times with 0.05% *m*-phosphoric acid and injected onto the HPLC system to measure total ASC.

2.4. Chromatographic conditions

The plasma ASC was separated on a Triacontyl (C_{30}) column, which gives a baseline separation of ASC from the *m*-phosphoric acid peak, whereas with the Octadecyl (Ultrasphere, C_{18}) column ASC appears in the tailing of the *m*-phosphoric acid, which may reduce the accuracy of the results. Liau *et al.* [2] also found the same problem at pH 2.55. In urine, the *m*-phosphoric acid concentration was low and

did not interfere with the ASC peak. Although it is possible to separate urinary ASC with the same column as used for plasma ASC, the less polar urinary constituents had a long (1 h) retention time. Therefore, urinary ASC was separated with an Ultrasphere column in order to reduce the analysis time to 20 min.

The mobile phase was identical for both plasma and urine analysis. Potassium dihydrogen phosphate, 13.61 g, was dissolved in 1 l distilled water, and acidified to pH 2.34 with concentrated *o*-phosphoric acid. Using this acidic mobile phase, the absorption maximum of ASC was found at 250 nm. The detector sensitivity was set to 1.0 AU and the signal output was 1 V. The 1-V signal was taken as full scale for the computing integrator system. The scale required for identification of the ASC peak and integration was selected through the software.

The flow-rate for plasma ASC measurement was set to 0.5 ml/min until the ASC had eluted (10 min). Thereafter, the flow-rate was doubled to the 15th min. For urinary ASC elution (8 min) the flow-rate was 0.5 ml/min, thereafter 2 ml/min for another 12 min. The Jasco pump was used to establish the flow gradient.

3. Results

3.1. Specificity

In order to confirm that only ASC was measured, and not some other substance, the ASC was oxidized with ascorbate oxidase strips dipped into the plasma and urine, respectively. Thereafter, no ASC peaks were seen at the ASC elution position. The result was reproducible on frequent repetition with samples from numerous individuals; for illustration see Fig. 1, A–C (plasma), and Fig. 2, A–C (urine). The ASC peak was quantified at wavelengths of 250 nm and 225 nm, respectively, both with standard solution and unknown samples. The peak-height ratio, or the absorbance ratio, at these two wavelengths was 1.91 (standard) and 1.86 (sample), which shows that ASC was indeed the light-absorbing substance at this elution position.

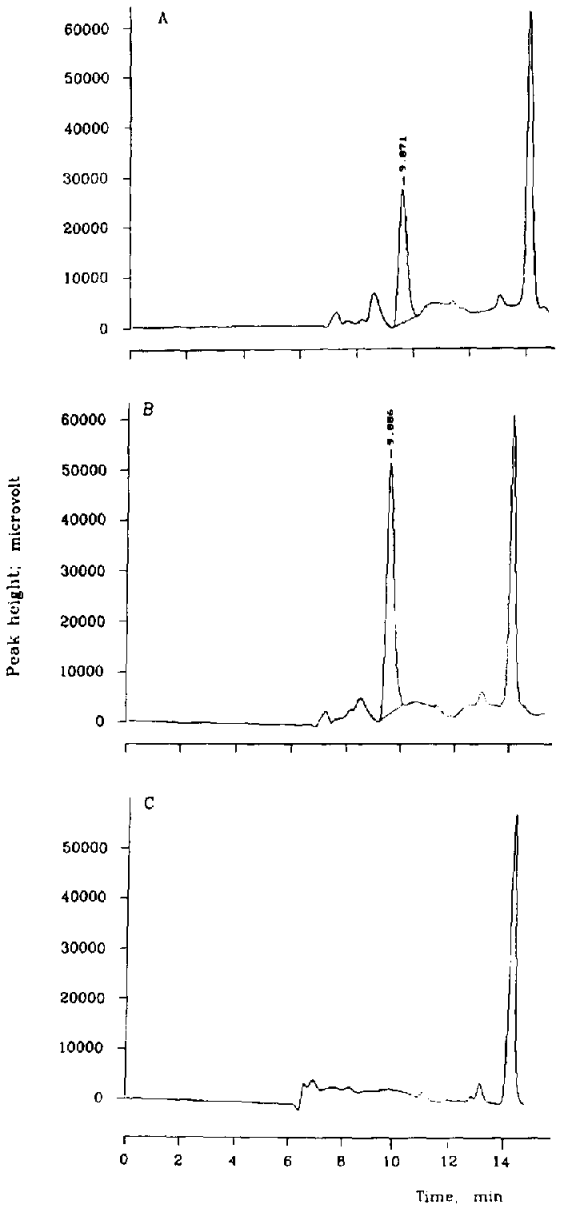

Fig. 1. Identification of ASC in plasma. Conversion of *y*-scale into absorption units (AU): 1 V = 1 AU. (A) Native sample, ASC retention time 9.9 min, concentration 9.3 mg/l. (B) Native sample of A, spiked with 8 mg/l ASC. (C) Sample of B, treated with ASC oxidase; note that the ASC peak seen in A and B has disappeared.

3.2. Recovery of ASC

Plasma samples were deproteinized with 10% *m*-phosphoric acid. In order to prove that ASC is

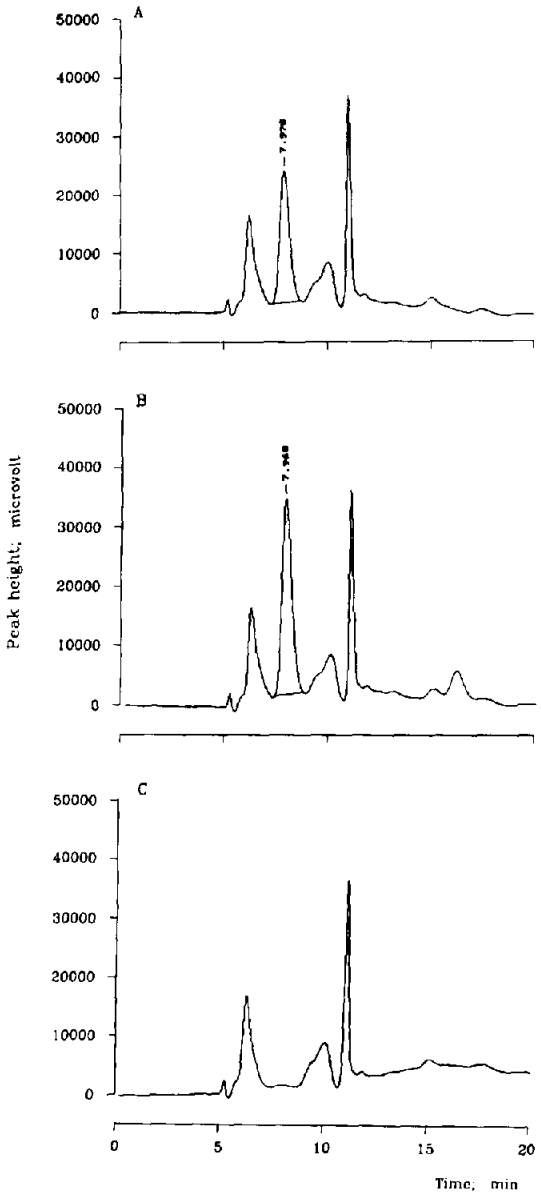

Fig. 2. Identification of ASC in urine. Conversion of *y*-scale into absorption units (AU): 1 V = 1 AU. (A) Pool urine sample, ASC retention time 8 min, concentration 81.3 mg/l. (B) Pool urine sample of A, treated with dithiothreitol, concentration of total ASC 120.9 mg/l. (C) Pool urine sample of A, treated with ASC oxidase.

not lost through the deproteinization step, aqueous ASC at a concentration 50 times higher than in plasma, was added in small volumes, for example 1:50, to give concentrations of total plasma ASC above the baseline of 4 and 8 mg/l. The amount recovered was taken as the difference between ASC in spiked and native plasma. On this basis, ASC recovery values were 3.99 ± (S.D.) 0.05 ($n = 5$) and 7.9 ± 0.07 ($n = 5$) mg/l, respectively.

3.3. Precision

The inter-assay variation of ascorbic acid concentration in pooled plasma ($n = 10$ replicates, measured on separate days) and standard solution ($n = 6$ replicates) was (mean ± S.D.; mg/l) 6.1 ± 0.07 (plasma), and 5.0 ± 0.04, 10.0 ± 0.04, 20.0 ± 0.06 (standards 5, 10, 20 mg/l, respectively); the corresponding C.V.s were (%): 1.1 (plasma), 0.8, 0.4, 0.3 (standards). The intra-assay variation of pool plasma was 6.1 ± 0.16 mg/l ($n = 10$), C.V. 2.6%.

The inter-assay variation for urinary ASC in a pooled sample was (mg/l) 81.3 ± 3.11 ($n = 10$), for total ASC in the same sample 120.9 ± 6.64 (C.V.s 3.8 and 5.5%, respectively). The intra-assay variation in urine was (mg/l) 81.3 ± 1.69 ($n = 6$) for ASC (C.V. 2.1%), and 121.1 ± 4.78 (C.V. 4.0%) for total ASC. The high portion of dehydroascorbic acid in the pooled sample is explained by uncontrolled exposure to air, which contrasts with the situation in fresh urine (see sample pretreatment). When analysing total ASC in individual urine samples, dithiothreitol was added immediately as described above, thereby preventing ascorbic acid oxidation and reducing the dehydroascorbic acid present. Increasing the dithiothreitol concentration in urine did not result in higher total ASC.

3.4. Linearity and sensitivity

For the substances already present in biological fluids, only external or aqueous standard calibration is possible. Plasma, either diluted or spiked with ASC, yielded values which correlated highly with those obtained from aqueous standard ASC ($r^2 = 0.999$), even when the plasma ASC concentration was above 100 mg/l. The sensitivity for plasma ASC was 2 ng on column.

For all measurements in plasma and urine the signal-to-noise ratio was restricted to 3.

For urinary ASC, linearity was examined up to 200 mg/l. When diluting samples with *m*-phosphoric acid 1:6 (v/v; see above), the lowest concentration which could be measured was 1.0 mg/l. Since it was possible to measure reliably down to 0.1 mg/l with a larger sample volume, the sample was minimally diluted with *m*-phosphoric acid. The detection limit for urinary ASC was 1.5 ng on column, which compares well with the sensitivity mentioned elsewhere [2].

With the methodology used for the determination of total ASC in urine, which involves immediate reduction of dehydroascorbic acid (see above), it was not possible to assess the completeness of the conversion of the oxidation product to ASC directly. However, on the basis of previous work showing that in the presence of 1 mmol/l dithiothreitol 100 mg/l of dehydroascorbic acid were completely reduced [11], the amount of dithiothreitol (100 mmol/l urine) used in our study should have been sufficient.

3.5. ASC in idiopathic calcium urolithiasis

In male and female stone patients (age 19–65 years, mean 42, $n = 80$), the median total ASC in 3 h fasting urine, which was snap frozen in liquid nitrogen within a few minutes after collection (see above), was 1.7 mg (range 0.04–27), in age-matched controls ($n = 60$) 3.7 mg (range 0.1–33). Owing to the large scatter of values (log–normal distribution) the medians of the two populations did not differ statistically.

The ASC concentration in fasting plasma – which is considered to reflect the vitamin C nutrition state [12] – in the two groups of participants in the study showed mean values (mg/ml) of 12.1 (patients; range 2.9–19.9) and 13.4 (controls; range 6.6–17.9). Again, these medians did not differ statistically.

4. Conclusions

The ASC method described may satisfy the requirements of modern clinical chemistry, in terms of specificity, accuracy, precision, sensitivity, and also the cost/efficiency ratio applicable to the study of a large series of samples. By appropriate pretreatment of urine the possibility that dehydroascorbic acid may not be detected, and that falsely low values for the vitamin status are obtained, is prevented.

The data for controls and stone patients are in agreement with those obtained using more complex HPLC methods [13,14], but disagree somewhat with those obtained using older methodology [10], probably due to the non-specificity of the color reaction in the latter. Moreover, plasma ASC concentration may be age-dependent, with higher values during childhood and adolescence [15], as compared with adulthood [16]. For renal stone research, the more important aspect may be the fact that ASC is probably normal in fasting plasma and urine of patients with metabolically active idiopathic calcium stone disease. On the basis of these and additional data on ASC from this laboratory [16] there may be a need to revise the widely held view that the amount of dietary ASC, intestinal absorption of ASC, disappearance half-life from plasma, or renal handling of ASC, may be substantially disordered in calcium stone disease [7,8], and that such factors may contribute to, or are responsible for, the increased oxaluria frequently observed in the latter.

5. Acknowledgement

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6. References

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