Journal of Chromatography, 391 (1987) 419–426
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 315

RAPID AND SENSITIVE DETERMINATION OF DEHYDROASCORBIC ACID IN ADDITION TO ASCORBIC ACID BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING A POST-COLUMN REDUCTION SYSTEM

S. J. ZIEGLER, B. MEIER and O. STICHER*

Pharmazeutisches Institut, Eidgenössische Technische Hochschule Zürich, CH-8092 Zürich (Switzerland)

(Received November 20th, 1986)

SUMMARY

An improved procedure for the direct determination of *l*-dehydroascorbic acid (DHAA), in addition to *l*-ascorbic acid (AA), has been developed. The two biologically active forms of vitamin C were separated using reversed-phase high-performance liquid chromatography. DHAA was reduced to AA with dithiothreitol (DTT) in a post-column reaction system. Complete conversion was achieved at 50°C using a 1-ml reaction coil. Recoveries were in the range of 95–99% and both forms could be detected spectrophotometrically at 267 nm with high sensitivity. Reproducible results [relative standard deviations 2.4% (DHAA) and 1.0% (AA), $n = 5$] were obtained when the method was applied to the analysis of rose hip samples.

INTRODUCTION

Owing to the similar biological activities of *l*-ascorbic acid (AA) and its oxidized form, *l*-dehydroascorbic acid (DHAA), the total vitamin C of a sample is the sum of both forms. It has been reported that methods that assay for only the reduced form of ascorbate in biological samples and foodstuffs may provide misleadingly low vitamin C values[1]. Various methods for the quantitative determination of AA have been proposed and several reviews of AA methodology are available[1–3]. A rapid and selective method for the assay of AA in rose hips has been developed previously in our laboratory[4].

The quantitative determination of DHAA, however, is still problematic. Analyses by currently available chemical methods are time consuming and susceptible to interferences with the matrix containing the vitamin. DHAA can be reduced to AA and subsequent analysis yields the total vitamin C. As most biological samples have much greater content of AA than DHAA, quantitation by difference has the disadvantage of being based on the measurement of a small increase over a large background.

A direct determination using high-performance liquid chromatography (HPLC) is possible[5], but lacks sensitivity owing to the poor UV absorptivity of

0021-9673/87/$03.50

DHAA even at low and, therefore, non-specific wavelengths. Keating and Haddad[6] and Baker *et al.*[7] were successful in enhancing the sensitivity by pre-column derivatization of DHAA with *o*-phenylendiamine (OPD); however, problems with the stability of the derivative in aqueous solutions were experienced. Vanderslice and Higgs[8] separated AA and DHAA using an anion-exchange resin, and fluorescent detection was achieved by a relatively complicated, two-step post-column reaction system involving oxidation of AA to DHAA followed by reaction with OPD. This resulted in significant peak broadening and a long run time of more than 30 min.

As a consequence of these limitations, we have developed a rapid and sensitive method for the direct determination of DHAA and AA. Further, we have applied this procedure to the analysis of rose hips in an effort to determine the total vitamin C content.

EXPERIMENTAL

Fig. 1 shows a schematic diagram of the HPLC system used. Isocratic analyses were performed with a Model 6000A pump, equipped with either a U6K injector or a Wisp 712 autosampler (all from Waters Assoc., Milford, MA, U.S.A.). Three different reversed-phase (RP) column materials were used, LiChrosorb RP-8 (5 μm), Nucleosil C_{18} (7.5 μm) and LiChrosorb RP-18 (5 μm), all with Knauer (Berlin, F.R.G.) column cartridges (250 × 4 mm I.D.). The pumping rate of the mobile phase (0.25% metaphosphoric acid) was 1.0 ml/min. For temperature control of the column and the reaction coil (Model URA 102, 1 ml internal volume) (Kratos, Ramsey, NJ, U.S.A.), a PCRS Model 520 heater system (Kratos) was used. The post-column reagent solution was delivered at a flow-rate of 0.5 ml/min by a URS 051 pumping

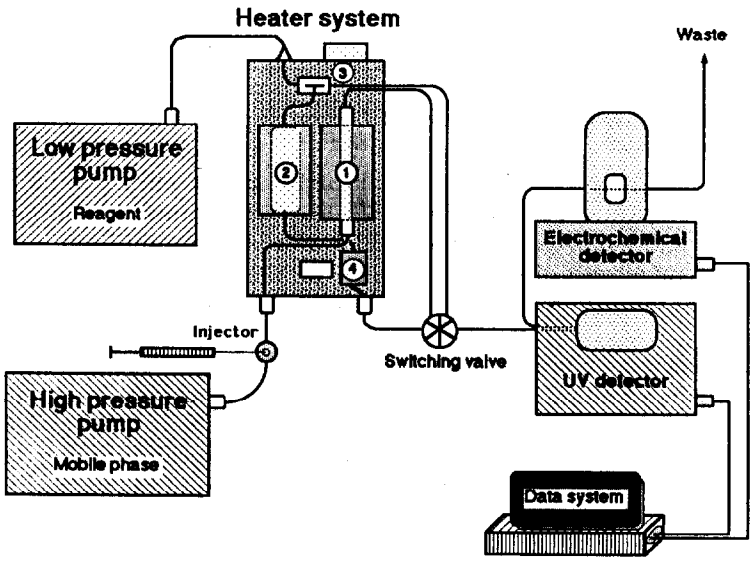

Fig. 1. Schematic diagram of the chromatographic system. 1 = Column; 2 = reaction coil; 3 = vortex mixer; 4 = heat exchanger.

system (Kratos). A Model 7000 switching valve (Rheodyne, Cotati, CA, U.S.A.) allowed the eluent to be routed either to the post-column reaction system or directly to the detectors. The mixed effluent was monitored at 267 nm either with an HP 1040 diode-array detector (Hewlett-Packard, Waldbronn, F.R.G.) or an LC 85 UV detector (Perkin-Elmer, Überlingen, F.R.G.) coupled in series with a Model 4B amperometric detector equipped with a glassy carbon electrode (BAS, West Lafayette, IN, U.S.A.). Owing to high background currents, working potentials above 400 mV *vs.* Ag–AgCl were avoided. For integration, either an SP Model 4000 data system (Spectra Physics, Santa Clara, CA, U.S.A.) or an HP DPU multi-channel integrator with an HP 85 computer was used.

Reagents

l-Ascorbic acid was purchased from Fluka (Buchs, Switzerland). The mobile phase was prepared by dissolving 2.5 g of metaphosphoric acid (Merck, Darmstadt, F.R.G.) in 1 liter of distilled water. The post-column reagent was prepared by dissolving 11.35 g of trisodium phosphate dodecahydrate, 6.65 g of sodium dihydrogen phosphate dihydrate and 150 mg of dithiothreitol (DTT) (all from Fluka) in 500 ml of distilled water, giving a buffered solution of pH 7.6. Mobile phases and reagents were membrane-filtered and deaerated by purging with helium prior to analysis.

Sample preparation

Standards. Stock standard solutions contained between 1 and 80 mg of AA per 100 ml of 1% metaphosphoric acid. AA and DHAA working standard solutions were prepared as follows: 3% bromine solution was added dropwise to 5.00 ml of the stock standard solution in order to oxidize AA to DHAA. Excess of bromine was completely removed by flushing with helium. After addition of a further 5.00 ml of the stock standard solution, the mixture was adjusted to a final volume of 20.00 ml with 1% metaphosphoric acid in a volumetric flask. Volumes of 10 μl of the solution obtained were injected into the chromatographic system or were processed through the clean-up procedure prior to analysis. Identical results were obtained with both methods.

Rose hip samples. Extraction with 1% metaphosphoric acid by means of a Polytron (Kinematica, Kriens, Switzerland) and sample clean-up using Bond-Elut C_{18} (3-ml disposable extraction cartridges) (Analytichem International, Harbor City, CA, U.S.A.) were performed as described previously[4].

Owing to the extreme instability of DHAA in aqueous solutions, the standards and samples cannot be stored for longer than 1 h even when refrigerated and, therefore, must be analyzed immediately after preparation. Calibration runs with freshly prepared working standard solutions were performed daily prior to the analysis of rose hip samples.

RESULTS AND DISCUSSION

Chromatographic separation of AA and DHAA

AA and DHAA could be separated on all the RP column materials used. The best results were obtained with the LiChrosorb RP-18 (5 μm) column owing to a distinctly stronger retardation of AA. The concentration of metaphosphoric acid in

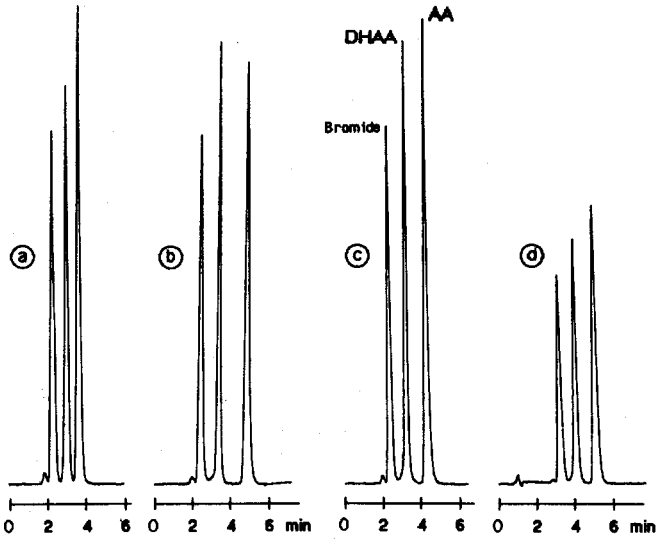

Fig. 2. Effect of the column temperature and of the reaction coil on the separation of DHAA and AA. Column: LiChrosorb RP-18 (5 μm), 250 × 4 mm I.D. Mobile phase: 0.25% metaphosphoric acid (1 ml/min). Injections of 10 μl of a solution containing 4.3 mg/ml of DHAA and 0.1 mg/ml of AA in 1% metaphosphoric acid. Detection at 225 nm. Column temperatures: (a) 20°C; (b) 40°C; (c) and (d) 27°C. Chromatograms a–c were obtained with the post-column reaction system switched off and d after post-column addition of 0.5 ml/min of water and passage through the reaction coil. Bromide (derived from the preparation of DHAA) is only detected owing to the high DHAA concentration needed for the direct spectrophotometric detection and the low wavelength employed.

the mobile phase (0.25%) was reduced from that recommended previously[4] in order to minimize the phosphate buffer concentration needed for the post-column neutralization step.

The influence of column temperature was evaluated over a range 10–50°C. The best separations were achieved at low column temperatures owing to a considerably stronger retardation of AA. Fig. 2a and b shows two examples of chromatograms obtained at 20 and 40°C, respectively. Unfortunately, DHAA elutes rather rapidly from RP columns. While the dead time was measured to be about 87 s (Nucleosil C_{18} column, sodium nitrate peak in water as the mobile phase), the maximum retention time for DHAA was 183 s. Although no difficulties were experienced during the analysis of the rose hip samples, a stronger retardation may be desirable in the analysis of other biological materials, particularly when interfering peaks occur.

Post-column reduction of DHAA

Okamura[9] has described a spectrophotometric method for the determination of AA and DHAA in which DHAA is completely reduced to AA by incubation with DTT. Owing to the rapid conversion, even at low temperatures, a modification of this method for use in a post-column reaction system showed promise. The reaction was found to be dependent on pH, temperature and reaction time. The reduction rate of DHAA by DTT reaches a maximum at pH 6.5–8.0[9]; therefore, the reagent was buffered in order to neutralize the strongly acidic mobile phase.

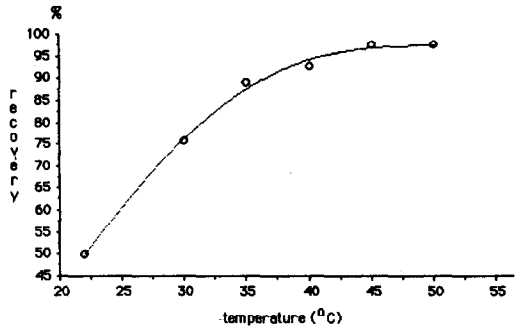

Fig. 3. DHAA reduction rate as a function of the reaction coil temperature.

Fig. 3 shows the relationship between reaction coil temperature and reduction rate. Complete conversion was achieved at temperatures above 45°C with a 1-ml reaction coil, resulting in a reaction time of about 50 s. Peak broadening was minimal and the separation of DHAA and AA was essentially unaffected. Fig. 2c and d show chromatograms obtained with and without the reaction coil.

The mixing of 0.5 ml/min of reagent with 1.0 ml/min of mobile phase caused only a minimal additional pulse, which primarely affected the baseline signal of the electrochemical detector. Almost no interference was observed when using the UV detector at a wavelength of 267 nm.

Detection

The UV absorption characteristics of AA depend strongly on pH. Whereas the absorption maximum at 242 nm in 0.5% metaphosphoric acid was used only for the detection of AA[4], the detection wavelength must be shifted to a higher value when working in a neutral pH range. Fig. 5 shows the UV spectra of both AA and the reduced DHAA taken on-line during a chromatographic run. The absorption maximum is at 267 nm. By using a photodiode-array detector, the identification and purity control of the peaks were greatly facilitated; the conversion of DHAA to AA can be readily validated by superimposing the spectra.

Although the electrochemical detector was superior with respect to sensitivity and selectivity, as described previously[4], our preliminary results now suggest problems such as high background current (originating from the oxidation of DTT) and electrode poisoning, resulting in impaired sensitivity with time and insufficient long-term reproducibility of the detector response. Ways of overcoming these problems have not yet been determined.

Recovery, reproducibility, linearity and sensitivity

The reaction conditions were optimized and the reliability of the method was evaluated by repeated injections of DHAA standards obtained by bromine oxidation as described previously. After post-column reduction, 95–99% (96.7 ± 1.6, n = 6 samples) of the original AA was recovered. Results were obtained by direct comparison of the peak area with that of simultaneously injected AA. The reproducibility of the reduction step was 1% [relative standard deviation (R.S.D.), n = 10 injections].

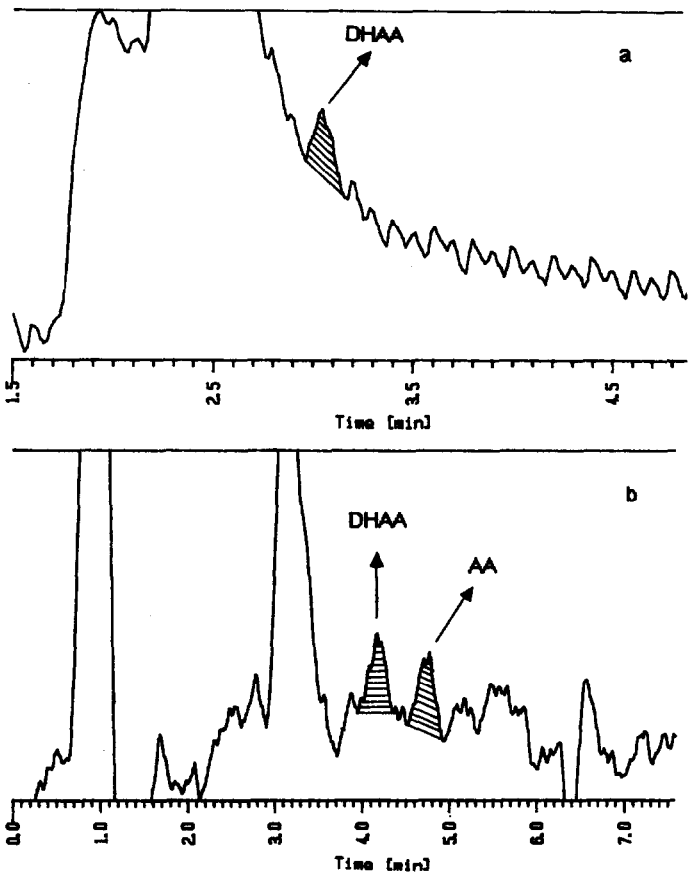

Fig. 4. Evaluation of the detection limits. Column: Nucleosil C_{18} (7.5 μm), 250 × 4 mm I.D.Other conditions as described in the text. (a) Direct detection at 225 nm. Injection volume: 10 μl. Amount injected: $2 \cdot 10^{-7}$ g. (b) Detection at 267 nm after post-column reduction of DHAA. Injection volume: 10 μl. Amount injected: $1.4 \cdot 10^{-9}$ g.

Incomplete conversion or loss of DHAA during the chromatographic run seems unlikely; pre-column reduction of DHAA with subsequent analysis for AA yielded identical recoveries. A small percentage of AA may be converted to diketogulonic acid and further breakdown products during the preparation of DHAA.

The relationship between peak area and concentration was evaluated over the range 0.0064–0.3825 mg/ml and found to be linear (n = 10, correlation coefficient r = 1 of AA and DHAA). The detection limit for DHAA by direct spectrophotometric detection at 225 nm of about $2 \cdot 10^{-7}$ g can be enhanced by a factor of more than 140 by using the proposed post-column system. The detection threshold for DHAA is as sensitive as that for AA and was determined to be $1.4 \cdot 10^{-9}$ g at a signal-to-noise ratio of about 2:1 (Fig. 4).

Assay of rose hip samples

The applicability of the system was demonstrated by the simultaneous deter-

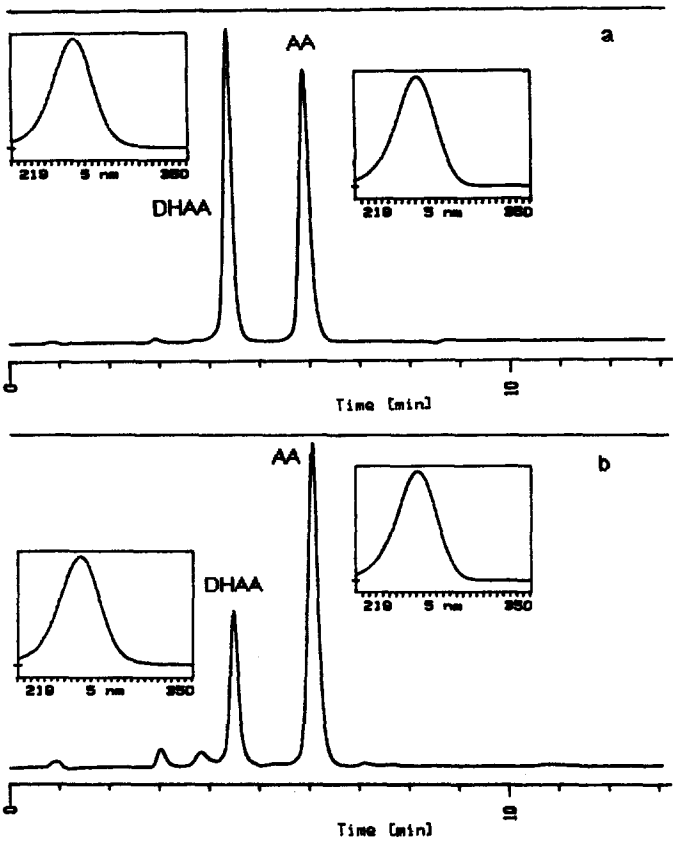

Fig. 5. HPLC elution profiles of (a) a standard solution and (b) a rose hip sample containing 29% DHAA of the total vitamin C content (0.67% dry mass). Column: LiChrosorb RP-18 (5 μm), 250 × 4 mm I.D. Column temperature: 19°C. Other conditions as described in the text. The UV spectra of AA and of the reduced DHAA were taken on-line during the chromatographic run.

mination of AA and DHAA in extracts of rose hips (Fig. 5). The precision of the assay method was checked by multiple analyses of a single sample. The R.S.D. for five measurements was calculated to be 1.0% for AA and 2.4% for DHAA. The sample contained 0.83% of AA and 0.16% of DHAA.

The recoveries of AA and DHAA in different standard solutions added to rose hip samples during sample preparation were found to be 100.1 ± 1.3% and 98.9 ± 1.9%, respectively (n = 5). These results indicate that the assay method has satisfactory accuracy.

As a result of the simultaneous determination of DHAA and AA, the analysis time is nearly the same as that for AA alone[4]. Whereas the chromatographic run time is about 7 min, sample extraction and clean-up, which can be done in triplicate or quadruplicate, require 15–20 min. Complicated and time-consuming pre-column derivatization procedures are not necessary. The use of an autosampler for full automation of the chromatographic system is limited by the labile nature of DHAA in aqueous solution. Although metaphosphoric acid proved to be an excellent stabil-

izing agent for AA, it is of little help with DHAA. A suitably effective alternative has not yet been found.

Results from analyses of various extracts, obtained either from fresh rose hips (frozen and lyophilized) or from commercial products, revealed considerably different vitamin C contents. DHAA values were in the range <1–29% of the total vitamin C content (0.1–1.3% dry mass). Significantly higher DHAA levels were found in lyophilized samples. It has not yet been established whether is a relationship between lyophilization and the DHAA to AA ratio or whether any of the drying procedures using heat are responsible for losses of DHAA.

CONCLUSION

In comparison with other analytical techniques, the method described here is superior with respect to sensitivity, selectivity, accuracy and analysis time. It takes advantage of (1) the high separation performance of RP-HPLC to resolve AA and DHAA, (2) the rapid and complete post-column reduction of DHAA by DTT and (3) the strong UV absorbance of AA. This procedure also proved to be reliable for the routine analysis of the total vitamin C content of rose hips. Moreover, it should also be applicable to the measurement of the DHAA and AA contents of other foodstuffs and biological materials.

REFERENCES

1 H. E. Sauberlich, M. D. Green and S. T. Omaye, *Adv. Chem. Ser.*, No. 200 (1982) 199.
2 M. H. Bui-Nguyen, in A. P. De Leenheer, W. E. Lambert and M. G. M. De Ruyter (Editors), *Modern Chromatographic Analysis of the Vitamins*, Marcel Dekker, New York, 1985, p. 267.
3 L. A. Pachla, D. L. Reynolds and P. T. Kissinger, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 1.
4 S. J. Ziegler, B. Meier and O. Sticher, *Planta Med.*, (1986) 383.
5 R. C. Rose and D. L. Nahrwold, *Anal. Biochem.*, 114 (1981) 140.
6 R. W. Keating and P. R. Haddad, *J. Chromatogr.*, 245 (1982) 249.
7 J. K. Baker, J. Kapeghian and A. Verlangieri, *J. Liq. Chromatogr.*, 6 (1983) 1319.
8 J. T. Vanderslice and D. J. Higgs, *J. Chromatogr. Sci.*, 22 (1984) 485.
9 M. Okamura, *Clin. Chim. Acta*, 103 (1980) 259.