Journal of Chromatography, 245 (1982) 355–358
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,003

Note

Differential determination of L-ascorbic acid and D-isoascorbic acid by reversed-phase high-performance liquid chromatography with electrochemical detection

C. S. TSAO* and S. L. SALIMI
Linus Pauling Institute of Science and Medicine, 440 Page Mill Road, Palo Alto, CA 94306 (U.S.A.)
(First received March 30th, 1982; revised manuscript received April 29th, 1982)

One of our vitamin C research projects requires a simple micro-method for the routine analysis of L-ascorbic acid (AA) and D-isoascorbic acid (IAA) in animal tissues and body fluids. IAA, a stereoisomer of AA and a commonly used food antioxidant, has about 5% of the antiscorbutic potency of AA[1]. These two isomers have been quantitatively differentiated by their different rates of osazone formation of the oxidized AA and IAA with 2,4-dinitrophenylhydrazine[2]. Paper chromatography[3–6] and thin-layer chromatography[7] have also been used for the separation of these two substances in microgram quantities. The recent high-performance liquid chromatography (HPLC) technique of Arakawa and co-workers[8,9] seems to be applicable to the estimation of AA and IAA in animal tissues, but the chromatograms presented by these authors show extraneous UV absorptions not related to either AA or IAA assay. AA and IAA in fruit juices have been separated by Bui-Nguyên[10] and by Geigert *et al.*[11] using HPLC and the chromatograms also show interfering UV-absorbing components. Moreover, these methods are not sensitive enough to serve our purpose.

HPLC has been used for the measurement of vitamin C with anion-exchange[12–16], LiChrosorb-NH_2 (refs. 8–10), reversed-phase C_{18} (ref. 14) and ion-pair reversed-phase C_{18} (refs. 17 and 18) columns and with electrochemical detection[12–17] or fixed-wavelength UV detection[8–11,18]. Ultramicro quantities of ascorbic acid in urine[15] and in plasma and leucocytes[16] have been analyzed using anion-exchange columns with electrochemical detection. Furthermore, Finley and Duang[18] have reported the use of paired-ion reversed-phase chromatography with UV detection for the simultaneous assay of ascorbic acid, dehydroascorbic acid and diketogulonic acid.

In the present study, reversed-phase HPLC with electrochemical detection is used and a suitable ion-pairing reagent is added to the mobile phase to couple with AA and IAA. The two structurally different complexes thus formed with the ion-pairing reagent are subsequently separated on the HPLC column. The method is reliable and highly sensitive and can be used to detect accurately as low as 2 ng of AA or IAA in a sample. The procedure is simple and rapid and involves only homogenization of the sample, centrifugation and direct quantitation by HPLC.

0021-9673/82/0000–0000/$02.75

EXPERIMENTAL

HPLC

The equipment and instrumentation were throughly described by Pachla and Kissinger[12,17]. All chromatograms were obtained using commercially available components and an amperometric detector (BioAnalytical Systems, West Lafayette, IN, U.S.A.). The analytical column was a 25 cm × 4.6 mm Ultrasphere ODS, 5 μm, Altex prepacked column. The eluent which consisted of 40 m*M* acetate + 1 m*M* decylamine in methanol–water (15:85) was partially degassed by a simple water aspirator before use. A Milton Roy pump (Model 396) equipped with a pulse dampener was used to pump the eluent through the system at a constant flow-rate of 0.93 ml/min. The injection valve was a Rheodyne injection valve (Model 7125). The detector electrode (BioAnalytical Systems) was packed with a wax-graphite paste, CP-W (BioAnalytical Systems). The potential of the chromatographic detector was set at 0.7 V *versus* an Ag/AgCl reference electrode. Quantitative determinations were made by comparing the peak heights of the samples with those given by known concentration of standards.

Reagents

The stock AA or IAA standard solution was prepared freshly with 50 m*M* perchloric acid and diluted immediately before use.

The stock acetate buffer (2 *M*) was prepared by diluting 101 ml of glacial acetic acid and 22.2 g of anhydrous sodium acetate to a total volumn of 1 l. The eluent buffer, 40 m*M* acetate, was prepared by mixing 20 ml of the 2 *M* stock buffer with 1 m*M* decylamine, 150 ml of methanol and an appropriate amount of deionized water to obtain a final volume of 1 l.

Sample preparation

At the time of analysis, the tissue was weighed while still cold. A 300-μl volume of 0.05 *M* perchloric acid was added to a 1.5 ml polypropylene tube containing 10–30 mg of tissue. The tissue was then thoroughly homogenized by sonication (Cell Disruptor, Model W-225 R, Heat Systems, Ultrasonic, Plainview, NY, U.S.A.). The homogenate was centrifuged at 1520 *g* for 10 min. A 5–10-μl volume of the clear supernatant thus obtained was injected directly into the liquid chromatographic column.

RESULTS

We have tested a number of ion-pairing reagents. They are brucine, strychnine, cinchonidine, quinidine, ephedrine, amphetamine, 1,5-dimethylhexylamine, decylamine and tridecylamine. All are acceptable ion-pairing reagents but the best separation is obtained with decylamine.

Fig. 1 illustrates the use of our method on tissue extract solutions obtained from mouse liver and brain. IAA and AA introduced as standards or present in the tissue extracts are well resolved under the experimental condition described in the Experimental section (Fig. 1 A2, B2, and C2). The ion-pair complex derived from IAA is more strongly retained on the column. A parallel experiment of tissue extract

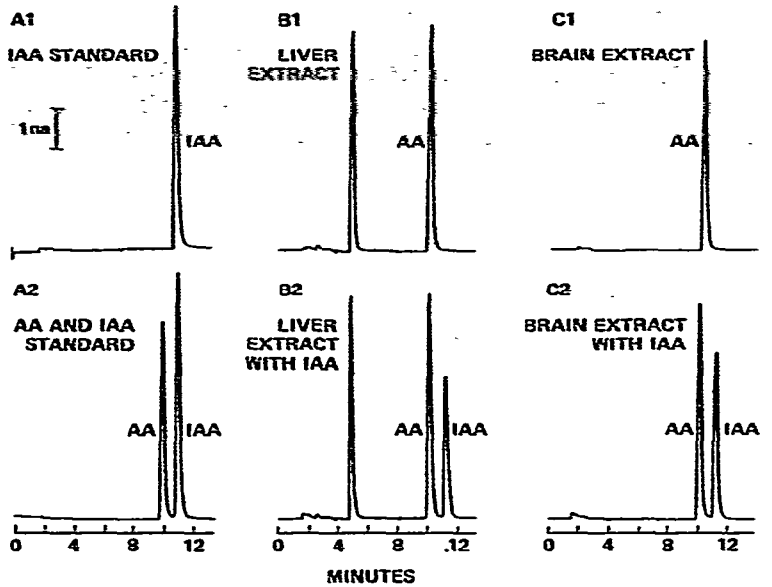

Fig. 1. Chromatograms for L-ascorbic acid (AA) and D-isoascorbic acid (IAA) in standards and in tissue extract samples of mouse liver and brain. (A1) 11 ng IAA standard injected; (A2) 11 ng IAA and 7 ng AA standard injected; (B1) liver extract containing AA and an unidentified substance; (B2) liver extract sample with 6 ng IAA standard addition; (C1) brain extract containing AA; (C2) brain extract sample with 7 ng IAA standard addition. Altex C_{18} reversed-phase 25 cm × 4.6 mm stainless-steel column. Mobile phase: 40 m*M* acetate buffer + 1 m*M* decylamine in methanol–water (15:85) at a flow-rate of 0.93 ml/min. Applied potential: 0.7 V *vs.* Ag/AgCl.

without IAA acid addition has been carried out for a chromatographic comparison (Fig. 1 B1 and C1).

When the liquid chromatography is run with a UV detector, the chromatograms of animal tissues[8,9] and fruit juiches[10,11] show the occurrence of interfering substances in the samples. The selectivity and sensitivity of the electrochemical approach become apparent when it is applied to these biological samples. Because of the requirement of electrochemical activity of the analytes, substances that oxidized at much greater potentials than the analyte would not be detected within the potential limits. Our results demonstrate that the electrochemical method is highly specific. The chromatograms of the brain tissues (Fig. 1 C1 and C2) are completely free of interfering substances. Fig. 1 B1 and B2 show an unidentified electrochemically active compound in the liver samples but it is well separated from the vitamin and its isomer by the HPLC column.

AA and IAA levels of mouse plasma and urine samples as well as orange juiche, milk, and animal food have been analyzed by our method. The chromatograms of these biological samples and food products resemble those shown in Fig. 1. The precision of this method was checked by multiple analyses on a single tissue sample containing IAA. Typically, the relative standard deviation for six measurements is calculated to be better than 4%.

ACKNOWLEDGEMENTS

We thank Drs. Linus Pauling, Emile Zuckerkandl, and Fred Stitt for their support, advice and suggestion. We would also like particularly to thank Mrs. Ruth Reynolds for providing us with literature research services during the course of this study.

REFERENCES

1 J. M. Rivers, E. D. Huang and M. L. Dodds, *J. Nutr.*, 81 (1963) 163.
2 O. Pelletier, *Can. J. Biochem.*, 47 (1969) 449.
3 T. Miki and Y. Sahashi, *Vitamins*, 25 (1962) 171 and 178.
4 C. E. Weeks and M. J. Deutsch, *J. Ass. Offic. Agr. Chem.*, 50 (1967) 793.
5 H. Kadin and M. Osadca, *J. Agr. Food Chem.*, 1 (1959) 358.
6 O. Pelletier and C. Godin, *Can. J. Physiol. Pharmacol.*, 47 (1969) 985.
7 G. S. Brenner, *J. Org. Chem.*, 29 (1964) 2389.
8 N. Arakawa, M. Otsuka, T. Kurata and C. Inagaki, *J. Nutr. Sci. Vitaminol.*, 27 (1981) 1.
9 M. Otsuka, T. Kurata, E. Suzuki, N. Arakawa and C. Inagaki, *J. Nutr. Sci. Vitaminol.*, 27 (1981) 9.
10 M. H. Bui-Nguyên, *J. Chromatogr.*, 196 (1980) 163–165.
11 J. Geigert, D. S. Hirano and S. L. Neidleman, *J. Chromatogr.*, 206 (1981) 396–399.
12 L. A. Pachla and P. T. Kissinger, *Anal. Chem.*, 48 (1976) 364.
13 K. V. Thrivikrama, C. Refshang and R. N. Adams, *Life Sci.*, 15 (1974) 1335.
14 R. Stillman and T. S. Ma, *Mikrochim. Acta*, (1974) 641.
15 K. Brunt and C. H. P. Bruins, *J. Chromatogr.*, 172 (1979) 37–47.
16 C. S. Tsao and S. L. Salimi, *J. Chromatogr.*, 224 (1981) 477–480.
17 L. A. Pachla and P. T. Kissinger, *Methods Enzymol.*, 62 (1979) 15.
18 J. W. Finley and E. Duang, *J. Chromatogr.*, 207 (1981) 449.