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Note

High-performance liquid chromatographic post-column reaction system for the electrochemical detection of ascorbic acid and dehydroascorbic acid

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Recently the simultaneous determination of ascorbic acid (AA) and dehydroascorbic acid (DHAA) in a variety of samples utilizing high-performance liquid chromatography (HPLC) has received considerable attention. This has ocurred to a large extent since Tolbert and Ward's[1] complaint in 1980 about a lack of a "completely satisfactory" assay for DHAA, their suggestions to reduce DHAA to AA, after separation, followed by ultraviolet (UV) detection or electrochemical detection (ED) and their belief in the importance of the DHAA/AA ratio.

Many detection schemes have been used. Some, with ED, determined AA followed by total AA and DHAA after reduction of the DHAA to AA with no separation[2,3]. Also UV detection was employed in a similiar manner[4–6]. Post-column derivatization followed by fluorescence detection has also been used[7–9]. The use of both ED for AA and UV detection for derivatized DHAA has been described[10,11]. Recent reviews have been published[12,13].

Ziegler *et al.*[14] reported a convenient HPLC post-column system in which DHAA is reduced to AA by dithiothrietol (DTT) followed by UV detection. The UV detection of DHAA in its reduced form, AA, is more sensitive and selective than UV detection of DHAA itself.

ED of both AA and DHAA (after reduction to AA) would be advantageous because it is more sensitive than UV detection and often more selective than UV and fluorescence detection. Ziegler *et al.*[14] also reported an unsuccessful attempt to use ED, attributing their difficulties to high background and electrode poisoning that resulted from the excess DTT. We report an extension of the system of Ziegler *et al.*[14] in which the excess DTT is reacted with N-ethylmaleimide (NEM), thereby permitting ED of both AA and DHAA. Okamura[15] did this in a non-chromatographic method.

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EXPERIMENTAL

Fig. 1 is a schematic diagram of the system. Numbers 1–10 refer to this figure. No. 1 is a Waters M-45 solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) with a solvent injection valve (Model 7010; Rheodyne, Cotati, CA, U.S.A.) equipped with a 20-μl loop. The analytical column (2) is a Nova-Pak C_{18} reversed-phase column from Waters.

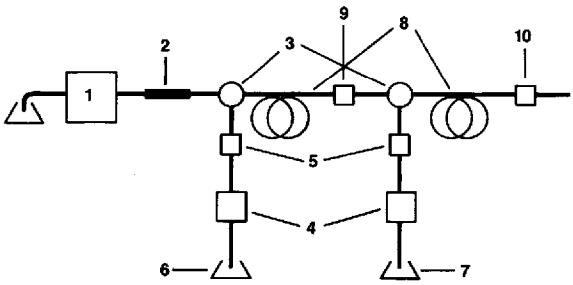

Fig. 1. Schematic diagram of reaction system. 1 = HPLC delivery system and sample injector; 2 = analytical column; 3 = mixing tees; 4 = pumps; 5 = pulse dampeners; 6 = DTT solution; 7 = NEM solution; 8 = reaction coils; 9 = UV detector; 10 = electrochemical detector. See text for details.

The mobile phase is 0.010 *M* $HClO_4$ and a flow-rate of 1.1 ml/min was used. Visco-mixer mixing tees (Lee Co., Westbrook, CT, U.S.A.) (3) are employed to combine the post-column reagents with the mobile phase. Reagent delivery pumps (4) are 350 pumps (SSI, State College, PA, U.S.A.) and pulse dampeners (5) are SSI LP-21.

The first post-column reagent (6) is 0.010 *M* DTT, 0.25 *M* NaH_2PO_4 and 0.25 *M* Na_2HPO_4, added at 0.50 ml/min. The second post-column reagent (7) is 1.0% NEM, 0.25 *M* NaH_2PO_4 and 0.25 *M* Na_2HPO_4, added at 0.60 ml/min. These reagents were stored under refrigeration for no more than a few days. Reaction coils (8) are 0.010 in. (0.254 mm) I.D. tubing of about 10 cm diameter. All tubing is stainles steel. The first reaction coil, in which DTT reduces DHAA to AA, is 24 m long. The second reaction coil, in which NEM reacts with the excess DTT, is 15 m long. The entire system was maintained at room temperature (22 $\pm$ 3°C).

A UV detector (9) (Waters Model 440 at 254 nm) was used for comparison with the electrochemical detector (10), an LC-3A Amperometric Detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) with an Re-1 Ag/AgCl reference electrode and a TL-3 glassy carbon working electrode. The glassy carbon electrode was pretreated each day by polishing with BAS polishing alumina (CF-1050, Bioanalytical Systems) followed by about 1 min of ultrasonic cleaning (Model SC-40; Sonicor, Copiague, NY, U.S.A.) and thorough rinsing with water and methanol. The working electrode was set at +0.60 V *vs.* the reference electrode. This potential was chosen because it was in the diffusion current region of hydrodynamic voltammograms obtained on this equipment.

DTT and NEM were obained from Aldrich (Milwaukee, WI, U.S.A.). All other reagents were A.C.S. reagent grade.

Standard AA solutions were made by dissolving the weighed reagent-grade compound in 0.010 *M* $HClO_4$. Standard DHAA solutions were made by oxidation of

AA solutions with saturated bromine water[15]. Excess bromine was removed by sparging with high-purity-grade nitrogen. AA and DHAA solutions of 10^{-6} *M* and higher (in 0.010 *M* $HClO_4$) were stable for at least 6 h. Standard solutions were prepared daily.

The mobile phase and post-column reactor solutions were filtered through 0.45-μm filters (Type HA; Millipore, Bedford, MA, U.S.A.) and vacuum deaerated.

RESULTS AND DISCUSSION

Standard solutions and selection of conditions

Fig. 2 shows examples of chromatograms of DHAA, AA and their mixture using the system described above. For the same concentrations the peak heights of AA and DHAA are essentially the same; this indicates, in accord with Ziegler *et al.*[14], that DHAA is completely reduced in the reactor (see data below). Background current due to oxidation of DTT is suppressed successfully by this system so that the electrochemical detector can be used at its most sensitive setting (1 nA full scale) with only small offset settings. Analyte concentrations as low as 10^{-7} *M* are easily determined (see below).

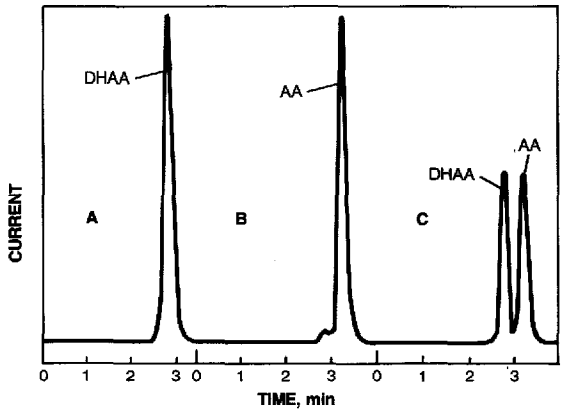

Fig. 2. Chromatograms of AA and DHAA. (A) 10^{-5} *M* DHAA; (B) 10^{-5} *M* AA; (C) $5 \cdot 10^{-6}$ *M* AA and DHAA.

Under he optimized conditions (described in the Experimental section), DTT was 3.1 m*M* in the first reaction coil and 2.3 m*M* in the second coil while NEM was 22 m*M* in the second coil. The pH was approximately 7. When the DTT concentration in the first coil was decreased to less than about 2 m*M*, DHAA was not completely reduced, as illustrated by these data:

DTT (mM)	*DHAA signal/AA signal*
3.9	1.04
2.7	1.04
2.1	0.94
1.5	0.88
1.3	0.85
0.83	0.36

The length of the first reaction coil was chosen as the minimum length yielding a ratio of 1 for the DHAA/AA signals using the optimized DTT concentration and 10^{-5} *M* DHAA samples. The NEM concentration, flow-rate and the second reaction coil length were chosen to give minimum background current on the electrochemical detector, along with acceptable broadening of the chromatographic peaks and an acceptable total pressure. The use of different chromatographic conditions may result in different optimum post-column reactor conditions.

Electrochemical detector drift

Carbon electrodes, in spite of their utility, are notorious for their changing sensitivity, "poisoned surfaces", and their varying behavior resulting from different pretreatments. A recent report[16] discusses this and shows how AA signals can vary with pretreatment of the electrode.

Fig. 3 shows how the sensitivity of the carbon electrode in this system decreases with time. This decrease in sensitiviy was not due to the loss of AA and DHAA in the standard solutions. This is clear because the UV detector (in the system as a check) gave the same signals over the course of the experiment. When the system was run without DTT and NEM, but otherwise the same, the sensitivity for AA changed considerably less for similar times.

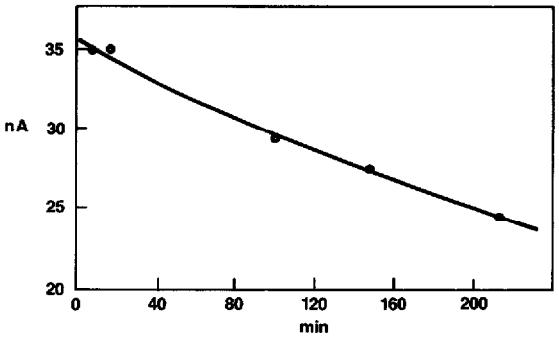

Fig. 3. Decrease in sensitivity with time. Signal obtained using 10^{-5} *M* AA and DHAA solutions.

Even though the residual current is sufficiently suppressed, apparently the DTT NEM system is slowly "poisoning" the electrode surface. The only noticeable change is in sensitivity; peak shapes remain the same. Chromatograms were obtained successfully with an electrode that had been used for about a week without cleaning and had lost approximately 90% of its original sensitivity.

This drifting sensitivity indicates that standards must be used along with each sample and, depending on the sensitivity required, the electrode must be cleaned at appropriate intervals. The length of these time intervals increases if the electrochemical detector is off between samples. The cleaning procedure, described above, can be completed in about 15 min. This pretreatment and the frequent use of standards is a minor disadvantage when the sensitivity and selectivity of the electrochemical detector are important.

Detection limits and linearity

Table I gives representative data which show that, after correction for the changing sensitivity of the detector, the peak height is linear with concentration of AA and DHAA from 10^{-5} to 10^{-7} *M* utilizing the entire sensitivity range of the instrument. The correction was made by multiplying each result by the ratio of the original sensitivity to the sensitivity at the time of measurement, obtained from a plot as in Fig. 3. The signals from repeat injections of 10^{-6} *M* and higher AA and DHAA solutions were reproducible to about 2%. The representative data in Table I have a relative sample standard deviation (coefficient of variation) of 3% ($n=6$) for the signal/concentration ratio of AA and 4% ($n=4$) for DHAA.

TABLE I

SIGNAL *VS.* CONCENTRATION

Representative data corrected for changes in detector sensitivity (see text).

DHAA		*AA*	
Concentration (M)	*Signal[a]/ concentration (mA/M)*	*Concentration (M)*	*Signal[a]/ concentration (mA/M)*
–	–	$1.33 \cdot 10^{-5}$	1.03
–	–	$1.33 \cdot 10^{-5}$	1.01
$1.10 \cdot 10^{-5}$	0.99	$1.10 \cdot 10^{-5}$	0.96
$1.10 \cdot 10^{-5}$	0.94	$1.10 \cdot 10^{-5}$	0.99
$5.50 \cdot 10^{-6}$	1.07	$5.50 \cdot 10^{-6}$	0.98
$1.10 \cdot 10^{-6}$	1.02	$1.10 \cdot 10^{-6}$	1.02
$2.20 \cdot 10^{-7}$	1.03	$2.20 \cdot 10^{-7}$	1.15
$1.10 \cdot 10^{-7}$	1.04	$1.10 \cdot 10^{-7}$	1.03
$1.10 \cdot 10^{-7}$	0.70	$1.10 \cdot 10^{-7}$	0.90

[a] Corrected as described in text.

The detection limit of $1 \cdot 10^{-7}$ *M* (about 3 × noise) was 10 times lower than the limit with the UV detector used for comparison purposes. This limit corresponds to detection of 0.3 ng of analyte; Ziegler *et al.*[14] report a detection threshold of 1.4 ng at a signal-to-noise ratio of about 2 with a UV system.

DHAA contaminant or inadvertent oxidation of AA

A small amount of DHAA (1–2%) was found in every sample of AA (see Fig. 2). The following indicate that the DHAA was a contaminant in the AA and not due to artifactual oxidation: (1) the DHAA peak remains in the same proportion to the AA peak upon serial dilution; (2) the DHAA peak remains constant with time; (3) the same peak is observed whether the AA is diluted with 0.010 *M* $HClO_4$ made from helium-sparged deionized water, air saturated deionized water or ordinary tap water; (4) the peak has the definite retention time of DHAA, unlike the flat, broadened signal obtained by Seki *et al.*[8] which was attributed to AA oxidation in the analytical column.

Disconcerting, however, are the facts that three different sources of AA, including the sodium salt of AA, gave essentially the same DHAA signal, and the most dilute (10^{-7} *M*) solution of AA did not always abide by finding point 1 above.

This problem will be investigated further but it is another illustration of the success of the post-column reaction system.

Examples of real samples

A few "real" samples were analyzed for DHAA and AA as illustrations of the use of this system. Fig. 4 shows chromatograms from a urine sample diluted 1 to 100 with 0.010 *M* $HClO_4$. Under these conditions the electrochemical detector is far superior to the UV detector because of its selectivity. This urine sample contained 0.36 m*M* AA and a trace of DHAA.

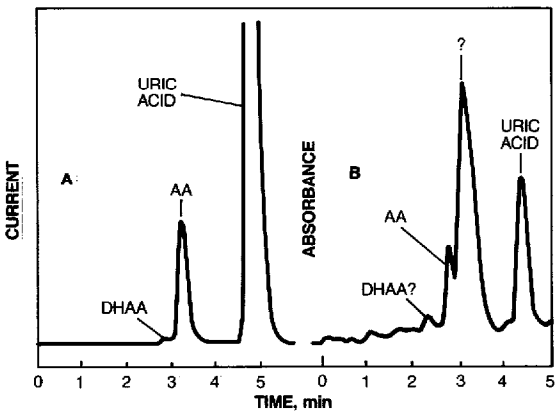

Fig. 4. Chromatograms of a urine sample. Sample diluted 1:100 with 0.010 *M* $HClO_4$. (A) ED; (B) UV detection.

A sample of rose hips tea made from a commercial brand contained 80 mg/l DHAA and 140 mg/l AA.

Saliva from one individual was found to contain 1.1 mg/l DHAA and only a trace amount of AA; a range of 0–3.7 mg/l of total AA in saliva has been reported[17].

CONCLUSION

The post-column reaction system using ED is an improvement over one previously described[14] which uses UV detection. ED affords greater sensitivity and selectivity than UV detection.

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