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Note

Liquid chromatographic determination of common water-soluble antioxidants in biological samples

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The most important low-molecular mass, water-soluble antioxidants in animal tissues are ascorbic acid (Asc), cysteine (Cys), reduced glutathione (GSH) and uric acid (UA). These antioxidants, or radical scavengers as they are also called, are part of the body's natural defence against free radicals and other oxidizing compounds, which are known to cause damage to cells in a variety of conditions, as discussed by Halliwell [1]. It is therefore of great interest to be able to measure antioxidants in body fluids and tissues. Although many authors have reported the determination of one or two of these antioxidants, no one has yet measured all four at once. This seems to be a necessary step to understanding their mode of action, because they are known to have mutual interactions [2]. As they are all easily oxidizable substances, electrochemical detection (ED), combined with high-performance liquid chromatography (HPLC), is an ideal method for their quantification. Asc and UA have been separated and measured without difficulty in serum [3], nerve tissue extracts [4] and human brain tumours [5] using a glassy carbon electrode. Determinations of the thiols GSH and Cys, which have been reviewed by Perret and Rudge [6], are more problematical. They require a high potential on the carbon electrode (Rudge et al. [7] reported an optimal potential of 1.8V) leading to lack of specificity, instability and rapid desensitization of the electrode. Various other electrodes operating at lower potentials have been developed, such as mercury [8], carbon paste containing cobalt phthalocyanine [9], gold–mercury amalgam [10], gold [11] and platinum [6]. After a comparison of results from commercially available gold and platinum electrodes, we chose the gold one [11] which, although it requires a higher potential than the gold–mercury electrode (0.5 V compared with 0.1 V) and is thus less specific,

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seems to be more stable for physiological samples [7]. Asc was also quantified at this electrode, whereas for UA a glassy carbon electrode (0.8 V) connected downstream was required. Sample preparation was minimal. Our system is suitable for the determination of water-soluble antioxidants in a variety of tissue extracts or body fluids. A short account of this method has already been published [12].

EXPERIMENTAL

Materials

The following compounds were used and were of the highest purity available: GSH, Cys, Asc, UA, ethylenediaminetetraacetic acid disodium salt (EDTA, Titriplex III) and *n*-octyl sodium sulphate from Merck (Darmstadt, F.R.G.); 1,4-dithioerythritol, N-acetyl-L-cysteine and DL-homocysteine from Fluka (Buchs, Switzerland); L-ergothionein from Serva (Heidelberg, F.R.G.); and cysteinyl glycine from Bachem (Bubendorf, Switzerland). Methanol for the mobile phase was chromatography grade (Merck), used as supplied. All solutions were prepared in water that had been doubly distilled in glass.

Chromatographic system

A high-performance liquid chromatography (HPLC) pump (420; Kontron Analytic, Zürich, Switzerland) was used in conjunction with an automatic sampler (WISP 710 B, Waters Assoc., Milford, MA, U.S.A.) and a membrane-type pulse damper (Portmann, Therwil, Switzerland). The analytical column was a 250 mm × 4.6 mm I.D. Supelcosil L-18, 5 μm average particle size (Supelco, Bellefonte, PA, U.S.A.) used at room temperature. Maintainance of the column at 25 °C as reported originally [12] was later found to be unnecessary, although it helped to stabilize retention times. The mobile phase, which was continuously purged with nitrogen gas, contained sodium dihydrogenphosphate (0.1 *M*) and 5% methanol. EDTA (0.1 m*M*) was added and the pH was set at 2.5 with orthophosphoric acid, two factors that helped to reduce the instability of the scavengers during chromatography and to improve peak shape and column efficiency [13]. *n*-Octyl sodium sulphate (1 m*M*) was used to increase the retention of the thiol compounds, enabling baseline separation [13,14]. The flow-rate, which was 0.5 ml/min with a new column, had to be reduced as the column aged. The analysis time was ca. 20 min. To improve the stability of the system the mobile phase was not recycled during chromatographic assays. The reservoir containing mobile phase was replenished daily during the course of an experiment and was completely renewed only when a series was completed. Two electrochemical detectors (Metrohm, Herisau, Switzerland) were connected in series, the first with a gold electrode set at 0.5 V for detection of Asc and thiol substances, and the other with a glassy carbon electrode set at 0.8 V for UA. Because of the relatively large amounts of antioxidants in the tissues investigated there was no need for high sensitivity (0.1 μA for both electrodes) or to reactivate the electrode regularly. In contrast to a previous report [11], we found that reactivation was positively harmful for the gold electrode, causing variability in the results. Data were collected and analysed on an IBM AT03 computer, with a data acquisition and con-

trol adapter to transform analog voltage signals from detectors into digital signals (Dynamic Solutions, Ventura, CA, U.S.A.). We used Maxima Chromatography software (Dynamic Solutions) for data acquisition and evaluation.

Sample preparation

As we found that antioxidant contents of samples were greatly diminished after overnight storage at 4 °C, centrifuged samples were kept for a maximum of 4 h before injection. To reduce oxidation, the homogenisation medium (0.5 *M* perchloric acid) was supplemented with EDTA (1 m*M*). Soft tissue (e.g. brain, spinal cord) was weighed and homogenized (1:10, w/v) with cold homogenization medium under nitrogen and with cooling in ice, using a micro-ultrasonic cell disrupter (Kontes, Vineland, NJ, U.S.A.). For tougher tissues (e.g. human breast) a different procedure was adopted: a fixed number of sections (25 μm) were cut from a frozen tissue cylinder of known diameter, giving a fixed mass of tissue that could be readily homogenized as described above (for details, see ref. 12). After homogenization the sample was centrifuged (4 °C, 12 000 *g*, 20–45 min depending on the tissue) to give a clear solution, and a portion of the supernatant (usually 10 μl) was injected into the HPLC system. Blood samples were collected into EDTA-coated tubes, diluted (1:10, v/v) with cold perchloric acid (1.0 *M* containing 1 m*M* EDTA) and treated as described above.

Quantification and computer evaluation

Using Maxima software, data can be collected from four independent systems, each with channels for up to four detectors. However, the concentration range that can be accurately integrated on one channel is rather limited and cannot be varied. On the other hand, the concentration ranges of antioxidants in tissue extracts vary greatly. As we used only two detectors, we were able to connect each one to two channels with different sensitivities, 0–1 V and 0–10 V, enabling accurate integration over a larger range.

Standard solutions of the antioxidants (Asc, GSH, Cys: 10^{-2} *M*; UA: 10^{-3} *M*) were prepared in sodium dihydrogenphosphate buffer (0.1 *M*, pH 2.5) containing EDTA (0.5 m*M*) and stored in portions at −80 °C. Before use, they were diluted as necessary with the same buffer, to give three to five concentrations covering those found in the extract to be analysed. Diluted solutions were kept at 4 °C and renewed every three to five days. The external standard method was used for quantification.

With the Maxima program, chromatograms, which after the run were stored on a hard disk, can be transferred to Lotus 1-2-3 or standard ASCII text files. Using the Turbo Pascal programming system (Borland) a 'data reduction' program was written to enable data needed for statistical calculations to be sorted out from a series of ASCII files and sent to a single file together with extra information if required. These new data files had a previously defined and programmed structure, i.e. a matrix with *N* chromatogram files and *M* variables. They can be evaluated using commercial software packages such as SYSTAT or BMDP statistical software or they can be transferred to another, more powerful computer such as a VAX 8800 for further data reduction or statistical evaluation.

RESULTS AND DISCUSSION

Using a reversed-phase system, which has been shown to be more efficient than ion-exchange for thiol compounds [13,14], we obtained satisfactory baseline separation. Fig. 1A and B show chromatograms from the gold and glassy carbon electrodes, respectively (both range 1 V full scale), for an extract of human cortex grey matter (1:10, w/v in our homogenization medium). An unidentified peak (X), which was present in relatively large amounts in many central nervous system samples and is probably a thiol, can be seen on the gold electrode only. Because of the low sensitivity of the carbon electrode there was no interference from other endogenous compounds of the central nervous system such as monoamines.

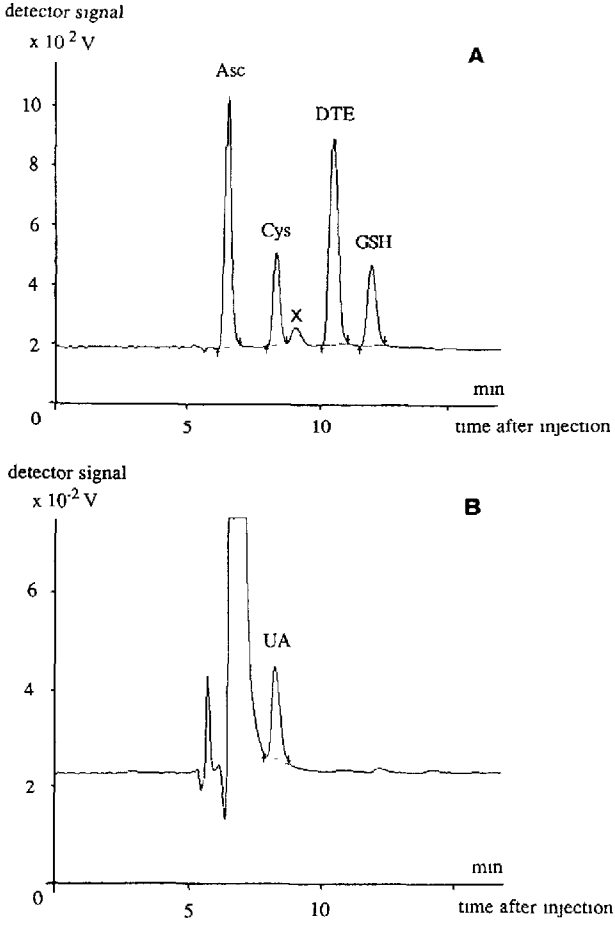

Fig. 1. Chromatograms of an extract (1.10, w/v, in 0.5 *M* perchloric acid containing 0.1 *M* EDTA) of human cortex grey matter. (A) Trace from gold electrode, 0.5 V. (B) Trace from glassy carbon electrode, 0.8 V. Range 1 V full scale X = unidentified peak, DTE = dithioerythritol. A 250 mm × 4.6 mm I.D. Supelcosil L-18 column was eluted at 0.45 ml/min with 0.1 *M* phosphate buffer (pH 2.5) containing methanol (5%), *n*-octyl sodium sulphate (1 m*M*) and EDTA (0.1 m*M*). Injection volume, 10 μl.

The gold electrode is relatively specific for thiols. A list of the retention times of the antioxidants and of certain naturally occurring thiol compounds that could interfere with their determination is given in Table I. Although the detection characteristics of the platinum electrode were very similar to those of the gold, there was always an unidentified interfering peak, even when distilled water was injected, which was not present with the gold electrode.

As can be seen from Fig. 1, with the packing material used we did not experience any of the problems described by Perret and Rudge [13], such as ghost peaks, multiple peaks, peak tailing and column binding. Column life was ca. six months when biological samples were injected. Regeneration by washing with methanol–water (50:50, v/v), as has been recommended [13], radically changed the column characteristics. We found that a mixture (50:50, v/v) of methanol with mobile phase buffer (pH 2.5) gave better results in our system.

The calibration curves for Asc and UA were linear. However, GSH and Cys both showed reduced peak response when higher amounts were injected (over ca. 2 nmol for GSH and 500 pmol for Cys), as reported by other authors [9]. In each case the Maxima program was able to fit the best curve. During the course of the day the response of the gold electrode was reduced to about three quarters of its value in the morning, returning to approximately the original level overnight, if nothing was injected. A similar phenomenon was reported by Kreuzig and Frank [11] and Perret and Rudge [13] who attributed it to the presence of an ion-pairing reagent. We compensated for this loss of sensitivity by calibrating both before and after the injection of samples and taking the average, as calculated by the Maxima program. In spite of these two difficulties, day-to-day variations were acceptable: Asc, 5.2%; UA, 9.4%; Cys, 7.0%; GSH, 6.8%; $n=8$.

Our original plan had been to use an internal standard, 1,4-dithioerythritol, to compensate for variations in electrode sensitivity. However, the size of the internal standard peak in samples, especially blood, was so variable that we abandoned

TABLE I

TYPICAL RETENTION TIMES OF COMPOUNDS DETECTED WITH THE GOLD ELECTRODE (0 5 V)

Chromatographic conditions as described in legend to Fig. 1; flow-rate, 0.5 ml/min.

Compound	Retention time (min)
Ascorbic acid	5.90
L-Ergothionein	6.97
Uric acid[a]	7.80
Cysteine	8 77
N-Acetyl-L-cysteine	12.09
Dithioerythritol	13.18
Glutathione	16.48
Cysteine glycine	19.72
DL-Homocysteine	20.03

[a]Uric acid was quantified with the glassy carbon electrode at 0.8 V.

the idea. This variation can probably be accounted for by thiol–disulphide interactions or by release of thiols from protein binding sites [6].

Recoveries from rat spinal cord homogenate (1:10, w/v, in our homogenization medium), provided that the samples were injected within 4 h after centrifugation, were: Asc, 97±2%; UA, 88±2%; Cys, 92±6%; GSH, 91±5%; $n=5$. Minimal detectable amounts (signal-to-noise ratio, 3:1) were ca. 0.2 pmol for Asc and UA, 1 pmol for Cys and 10 pmol for GSH, values comparable with those found by Stein et al. [15] for Cys and GSH.

With the method described here a concomitant measurement of the oxidized forms of the antioxidants, e.g. glutathione disulphide, is not possible. Various authors [15–17] have described methods in which thiols are quantified before and after the application of a high reducing potential (−1.0 V). This enables the proportions of the oxidized and reduced forms of radical scavengers to be determined. A modification of our method to include such a prior reduction should be possible.

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