Analysis of water-soluble antioxidants by high-pressure liquid chromatography

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The measurement of endogenous substances that function as biological antioxidants is of importance because the values obtained might be an index of future health. We quantified three water-soluble antioxidants by high-pressure liquid chromatography with electrochemical detection (h.p.l.c.-e.c.). Current-voltage relationships made at various settings of the D2 porous graphite electrode help to identify ascorbic acid, glutathione and uric acid. The antioxidants are determined simultaneously and

without need for derivatization. The method is seen to be useful for comparison of normal rat liver with liver that had undergone oxidative stress through ischaemia. Antioxidant levels in liver, kidney, pancreas and intestinal mucosa are presented and compared with literature values. Endogenous contents of oxidized forms of ascorbic acid and glutathione become apparent following exposure of tissue samples to a strong reductant such as 2-mercaptoethanol.

INTRODUCTION

Biochemicals that have antioxidant properties are receiving wide attention because of their possible roles in health maintenance. The endogenous level of one or more antioxidants might reflect, or be predictive of, tissue disease. Thus assays that combine a high level of sensitivity, accuracy and convenience for these substances have considerable application. Because water-soluble antioxidants have interrelated biological functions on a molecular level [1], it would be helpful to determine the level of several antioxidants in a single assay.

The metabolic role of low-molecular-mass antioxidants generally involves their participation in some form of redox biochemistry. Ascorbate and urate are thought to scavenge reactive free radical species that result from radiation, oxygen metabolism, carcinogen metabolism, etc. This reduces the radical's potential for toxicity, and results simultaneously in oxidation of the scavenger to a form that is no longer effective as an antioxidant [2–5]. GSH functions both by eliminating hydrogen peroxide, a free radical precursor, and by serving as a reducing cofactor in enzymic reactions of other antioxidants [6].

The concept is emerging that the body generally remains in a state of health as long as antioxidant defences are sufficient to prevent radicals from interacting with DNA, membrane lipids, etc. Tissue damage and disease would occur when the body has impaired ability to maintain its antioxidants in the proper form. The ratio of the reduced form to the total (reduced plus oxidized) form is referred to as the redox state [7]. Ascorbate oxidizes to dehydro-L-ascorbic acid, urate to allantoin and GSH to GSSG. Our approach could be useful in evaluating a concept reviewed previously [8] that the relative amounts of oxidized and reduced forms of major antioxidants in the body might be either a determinate or a useful indicator of disease.

We report herein the application of high-pressure liquid chromatography with electrochemical detection (h.p.l.c.-e.c.) to quantification of three water-soluble low-molecular mass antioxidants. Extracts of liver, kidney, pancreas and intestinal tissue are used to demonstrate how the method can be applied to biological samples; other applications will probably require modifications. The present goal was not to present extensive data

on tissue redox state but to show how h.p.l.c.-e.c. can be used to collect such information on extracts of representative tissues. Although existing applications are useful for either glutathione [9–11], ascorbate [12] or uric acid [13,14], little effort has been made to obtain simultaneous assays. One method was presented [15] that combines the high selectivity of electrochemical detection with some advantages of reversed-phase chromatography; we described changes in electrodes, stationary phase and mobile phase that allow improved performance.

MATERIALS AND METHODS

Reagents

Phosphoric acids were obtained from Fisher; uric acid, ascorbic acid, 2-mercaptoethanol, GSH and GSSG were obtained from Sigma.

Apparatus

The liquid chromatograph was modified slightly from Bode and Rose [12]. It consisted of a Beckman Model 110B solvent pump, Waters 710B injector, Waters Resolve C18 Guard-Pak pre-column, Waters RCM 8 × 10 Module and Resolve reverse-phase C18 column. The detector was an ESA Coulochem Model 5100A with a Model 5010 analytical cell that makes use of two porous graphite electrodes. The output of the detector was calibrated in arbitrary units of current and integrated on a Waters 745 Data Module; commercial standards of each antioxidant were run routinely. The optimal mobile phase was determined in preliminary studies to be 0.2 M $\text{KH}_2\text{PO}_4/\text{H}_3\text{PO}_4$ at pH 3, delivered at 1 ml/min. Detector settings for various applications are described below.

Tissue processing

Tissue was obtained from healthy decapitated adult rats. They were maintained at 0 °C through the following procedure. Pieces of approximately 0.5 g were weighed, minced and homogenized under N_2 with a Teflon pestle in 50 mM H_3PO_4 (pH 1.8) that contained 0.1 mM EDTA to minimize autoxidation. The pH of

the resulting extract was below 2.0. The extract was spun for 2 min at 16000 *g*. One aliquot of the supernatant was ready for injection on to the chromatograph; another aliquot was added to an equal volume of 200 mM potassium phosphate (pH 9.0). The remaining mixture had a pH of ~ 7 , and was treated with 10 mM 2-mercaptoethanol at 23 °C for 35 min before injection. The tissue-sampling technique was validated by the use of internal standards run on a sample of tissue adjacent to that described above. A known amount of each antioxidant was added to the homogenization medium prior to introducing the minced tissue, and the percentage recovery of each internal standard could be determined. Endogenous tissue concentrations are based on a common value of tissue water being 80 % of tissue wet weight [16].

The approach above was used to make a comparison of normal and ischaemic liver on a pair of male rats. Rats were anaesthetized with 60 mg/kg sodium pentobarbital administered intraperitoneally. Ischaemia was induced in one rat by a 15 min ligation of the hepatic artery and portal vein. In the control rat, the liver was removed after 15 min of anaesthesia without ligation. Livers from control and ischaemic rats were processed as above. For comparison with tissue antioxidant levels determined by h.p.l.c.-e.c., previously described levels of ascorbic acid and uric acid were determined by absorbance at 280 nm [14] and GSH was determined by the enzymic method based on the catalytic action of the GSH reductase system [17].

RESULTS AND DISCUSSION

Analysis of standards

The detector's response to each antioxidant of interest was determined by making repeated injections of standards on to the chromatograph. From preliminary evaluations the upstream detector D1 was normally maintained at -0.1 V; the downstream detector D2 was set at various potentials from $+0.4$ to $+0.8$ V. Figure 1 shows the amount of current required by the detector to oxidize ascorbate, urate and GSH at several D2

Figure 1 Current-voltage curves of antioxidants by h.p.l.c.-e.c.

Peak area of current response is plotted against the voltage setting of the D2 electrode. Ascorbate was injected (1.3 nmol; Δ), GSH (13.3 nmol; $\circ$) and uric acid (1.6 nmol; $\bullet$). Each substance was dissolved in 10% phosphoric acid. Mobile phase was 0.2 M potassium phosphate buffer (pH 3.0) delivered at 1 ml/min. Injection volume was 20 μ l.

Figure 2 Standard curves from h.p.l.c.-e.c. determination of antioxidants

Conditions were as in Figure 1, with the D2 electrode set at 0.5 V.

Figure 3 Chromatogram of antioxidant standards dissolved in saline and stabilized in phosphoric acid

Retention times (in min) are: solvent front, 2.6; ascorbate, 5.6; GSH, 8.1; and uric acid, 13.2. For reference, a decay product of glutathione (cysteine, 3.2 min) and uric acid (allantoin, 11.4 min) are shown.

settings. It is apparent that good sensitivity for ascorbic acid and uric acid is reached at a relatively low setting of D2, whereas maximal sensitivity for GSH might be possible by raising D2 to a higher voltage. We did not routinely operate with D2 above 0.6 V, however, because the background became excessive (see e.c. detection of GSSG below). A standard curve run for each antioxidant obtained from a commercial source shows that a linear relationship exists between the quantity injected on to the column and the response (Figure 2). Figure 3 is a demonstration of the separation of several commercial antioxidants.

Analysis of biological samples

We evaluated samples of liver, kidney, pancreas and intestinal

Table 1 Antioxidant levels in some rat tissuesValues are means $\pm$ S.E.M. of two observations on three animals.

Tissue	Level (mmol/litre)		
	Ascorbic acid	GSH	Uric acid
Liver	4.3 ± 0.52	5.2 ± 0.31	0.10 ± 0.01
Kidney	2.6 ± 0.33	3.0 ± 0.18	0.32 ± 0.01
Pancreas	0.26 ± 0.47	0.49 ± 0.02	0.19 ± 0.01
Colon	3.3 ± 0.15	0.21 ± 0.01	0.89 ± 0.05

Figure 4 Chromatogram of rat liver extracts

Upper trace: the tissue was homogenized at 0 °C under N_2 in phosphoric acid that contained 0.1 mM EDTA. After centrifugation at 16000 g , a 20 μ l aliquot of the supernatant was injected on to the h.p.l.c. column. Retention times (in min) are: solvent front, 2.8; ascorbic acid, 5.6; GSH, 7.7; uric acid 13.2; Lower trace: rat liver after 15 min of ischaemia, then processed as above. Conditions as in Figure 2.

mucosa from rats. The mean values we calculate for antioxidant content are shown in Table 1 and are in the range of those previously reported for rodent liver ascorbic acid [18], glutathione [9] and uric acid [14,19]. The coefficient of variation (mean divided by S.E.M.) in each case is quite adequate, considering the small number of observations. Internal standards prepared as in the Materials and methods section were evaluated in liver samples in triplicate for each antioxidant. Our mean values of recovery ($\pm$ S.E.M.) were generally similar to values in the references cited: ascorbic acid, $94 \pm 6\%$ [14,15]; glutathione, $97 \pm 8\%$ [10,15] and uric acid, $95 \pm 6\%$ [14,15]. The results of parallel studies performed on the basis of absorbance at 280 or 412 nm

Figure 5 The tissue was extracted in weak phosphoric acid (pH 2.0) and then neutralized in 200 mM potassium phosphate

(b) An aliquot was injected on to the column. (a) The extract was exposed to 2-mercaptoethanol at 23 °C prior to injection. The 8% greater peak area at 5.3 min indicates that some of the oxidized product of ascorbic acid was present in the extract. The peak at 10.1 min is 2-mercaptoethanol.

indicated that the present approach gives results generally 5–13% lower than earlier methodology. This might be attributed to the less specific nature of spectrophotometric assays as compared with electrochemical detection.

The separation between ascorbate, GSH and uric acid is evident from injection of a liver extract (Figure 4). It is significant that each peak returns essentially to base line before the start of a new peak; this is frequently not the case with h.p.l.c. when several related compounds are injected in a single sample. A comparison is made with ischaemic liver tissue from a different rat. The level of ascorbic acid in liver of that animal was 81% lower, glutathione was 65% lower and uric acid was not different.

2-Mercaptoethanol was previously demonstrated to bring about quantitative reduction of the oxidized form of ascorbic acid [12]. In samples of normal rat liver there was little increase in the peak areas brought about by exposure of the sample to 2-mercaptoethanol. Only the concentration of ascorbic acid consistently increased ($8.9 \pm 0.5\%$, $P < 0.05$; Figure 5). In a current evaluation with commercial GSSG we found that this procedure also provides nearly quantitative reduction of GSSG ($94 \pm 5\%$; $n = 4$) in an homogenate of rat liver; this can be used to quantify

Figure 6 Electrochemical properties of the peaks at retention times from Figure 4 compared with peaks from commercial ascorbic acid (triangles), GSH (squares) and uric acid (circles)

The potential of the D2 electrode was varied as the peak area for the homogenate (closed symbols) and the commercial standards (open symbols) were determined. All values are expressed as a ratio of that obtained at 0.5 V.

Figure 7 Solid lines show analysis of GSH (filled) and GSSG (open) with electrode D2 settings of 0.5–0.8 V

Dashed lines stimulate the expected responses from GSH and GSSG at D2 settings of 0.8–1.0 V based on information distributed by the detector's manufacturer.

GSSG. We were not successful in bringing about quantitative reduction of the oxidation product of uric acid with 2-mercaptoethanol, and suggest another approach might be more suitable. Tissue concentrations of reduced and oxidized forms of glutathione and ascorbic acid in various tissues and body fluids have been published previously [9,11,20]. The application of this methodology to tissues that have undergone oxidative stress will be presented in a separate report (R. C. Rose et al., unpublished work).

It was observed in Figure 1 that each antioxidant has a unique current–voltage relationship. This can be made use of to help confirm the identity of a particular peak from a biological sample. Thus, a current–voltage curve was created for both a

liver sample and for a commercially acquired form of what the peak of interest is anticipated to be. For example, the tissue-sample peak that appeared in Figure 3 at a retention time of 12.2 min was thought to be uric acid, as the uric acid standard on that day had a retention time of 12.28 min. The extract of liver and the various standards were repeatedly injected as the potential of the D2 electrode was altered. It is seen in Figure 6 that the curves for tissue-sample peaks and standards of uric acid, glutathione and ascorbic acid were indistinguishable.

An additional evaluation of GSSG can be made by setting D2 higher than we normally do. A response was seen between 0.7–0.8 V, although the sensitivity was much lower than for GSH (Figure 7). Information distributed by the manufacturer of the detector indicates that the responses at still higher D2 settings would be approximately as the dashed lines indicate. The utility of this approach is indicated by the results of making a second injection on to the h.p.l.c. of the ischaemic liver preparation from Figure 5, this time with D2 set at 0.8 V. The results are used to make a second calculation of the glutathione redox state; in this instance 9.4% was in the oxidized state, which confirms the value of 8.3% (after 2-mercaptoethanol reduction) from the liver extract above. We did not pursue this because with our mobile phase the background with D2 at 0.8 V had increased to > 20-fold that seen at 0.4 V. Although the higher background became quite stable and did not necessarily interfere with the assay (see also Figure 4 in [9]), the manufacturer advised that routine use would result in a shortened useful life of the analytical cell. It is possible that by selecting an alternative mobile phase, good sensitivity for GSSG can be obtained without excessive background.

The present work demonstrates that h.p.l.c.–e.c. can be used to quantify in a single injection the reduced form of three prominent tissue antioxidants. By making a second injection of the tissue extract subsequent to exposure to a strong reductant, endogenous levels of total vitamin C and glutathione can be obtained. Thus, an evaluation of redox state is available, as in the present example for ischaemic liver tissue. The choice between reduction by 2-mercaptoethanol and a high D2 voltage setting must be made according to the specific conditions of the study. Other groups have also reported that normally most tissue ascorbic acid and glutathione are in the reduced state [9,20]. It is acknowledged that other tissues in health and disease have different properties and this, or any approach, might have to be adapted. In particular, there must be concern about how stable each antioxidant is during extraction. The current practice of immediate tissue extraction into cold, deoxygenated acid with EDTA appears to preserve the antioxidants of interest better than the more lengthy derivatization processes. *N*-Ethylmaleimide [10] and numerous other compounds have been used to minimize autooxidation of endogenous compounds during processing. It will be necessary for each application to show that the compounds being assayed are not altered. We accomplished this by demonstrating that a known amount of each compound added to the homogenizing fluid prior to tissue extraction is quantitatively recovered; alternatively, a tracer amount of radio-labelled antioxidant could be added to the homogenizing buffer and assayed subsequently for molecular form. This problem needs to be addressed because it is not specific to h.p.l.c.–e.c. The somewhat lower values reported herein for tissue antioxidants might be attributed to the less specific nature of previous spectrophotometric assays that can respond to a variety of substances at any given wavelength.

Two perceived strengths of this application are that it is based on an h.p.l.c. column and mobile-phase system that allow separation of antioxidants and it incorporates the specificity of

electrochemical detection. A comparable h.p.l.c. assay based on absorption detection at 280 nm was applied to quantification of uric acid in human lens; the chromatogram had at least 15 discernible peaks, several of which had prominent shoulders [21]. The trace in that study rarely reached baseline, in contrast to Figure 4. The absence of a large number of potentially interfering peaks in the present application allows one to distinguish substances of interest. An earlier h.p.l.c.-e.c. system [9] was suitable for analysis of glutathione alone but had poor separation with a mixture of antioxidants. Others [15] made use of h.p.l.c.-e.c. that incorporated one gold electrode and one glassy carbon electrode. Disadvantages of this were that one chromatogram was required to quantify uric acid and another for ascorbic acid and GSH. Also, the response to the gold electrode routinely varied ~ 25% during the day. The analytical column developed back pressure that limited flow rate to 0.5 ml/min with a new column and required a still lower flow rate as the column aged. No evaluation of redox state was made in that study. Several differences in the present approach allow simultaneous quantification of physiologically active antioxidants.

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