[20] Determination of Oxidized and Reduced Lipoic Acid Using High-Performance Liquid Chromatography and Coulometric Detection

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Introduction

α-Lipoic acid, also known as thioctic acid, 1,2-dithiolane-3-pentanoic acid, 1,2-dithiolane-3-valeric acid, or 6,8-thioctic acid, has generated considerable clinical interest as a cellular thiol-replenishing and redox-modulating agent.[1-5] Biologically, lipoate exists as lipoamide in at least five proteins where it is linked covalently to a lysyl residue. Four of these proteins are found in the α-keto acid dehydrogenase complex, the pyruvate dehydrogenase complex, the branched chain keto acid complex, and the α-keto dehydrogenase complex. The fifth lipoamide moiety is in the glycine cleavage system.[6] The mitochondrial E3 enzyme, dihydrolipoyl dehydrogenase, reduces lipoate (LA) to dihydrolipoate (DHLA) at the expense of NADH. The enzyme shows a marked preference for the naturally occurring *R*-enantiomer of LA.[7] Lipoate is also a substrate for the NADPH-dependent enzyme glutathione reductase.[8] It has also been found that thioredoxin reductase from calf thymus and liver, human placenta, and rat liver reduces both LA and lipoamide efficiently.[9]

To develop an understanding of the mechanisms involved in the biological functions of supplemented LA, much of the current interest is focused on the fate of exogenous LA in cultured cells, as well as in animal and

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[2] C. K. Sen, *Biochem. Pharmacol.* **55,** (1998).
[3] C. K. Sen, S. Roy, and L. Packer, *in* "Therapeutic Potential of the Antioxidant and Redox Properties of α-Lipoic Acid" (L. Montagnier, R. Olivier, and C. Pasquier, eds.), p. 251. Dekker, New York (1997).
[4] L. Packer, S. Roy, and C. K. Sen, *Adv. Pharmacol.* **38,** 79 (1997).
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[6] K. Fujiwara, K. Okamura-Ikeda, and Y. Motokawa, *FEBS Lett.* **293,** 115 (1991).
[7] N. Haramaki, D. Han, G. J. Handelman, H. J. Tritschler, and L. Packer, *Free Radic. Biol. Med.* **22,** 535 (1997).
[8] U. Pick, N. Haramaki, A. Constantinescu, G. J. Handelman, H. J. Tritschler, and L. Packer, *Biochem. Biophys. Res. Commun.* **206,** 724 (1995).
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human tissues. Both LA and its reduced form DHLA have remarkable antioxidant properties.[10] Both of these compounds have been reported to regulate a number of critical cell functions such as biosynthesis of reduced glutathione (GSH), recycling of other oxidized antioxidants, regulation of cellular reducing equivalent homoeostasis, and regulation of agonist-induced activation of equivalent homeostasis, and regulation of agonist-induced activation of transcription factors, gene expression, and apoptosis.[11] To understand how LA and DHLA regulate various aspects of cell function, it is important to be able to detect LA and DHLA accurately in biological samples. Conventional high-performance liquid chromatography (HPLC) detection using ultraviolet or fluorescence detection methods are not feasible because both LA and DHLA lack adequate chromophores. Another detection method that has been used widely is based on gas chromatography and mass spectrometry. This method is highly sensitive; however, the major limitation of this method is that it cannot distinguish between oxidized and reduced forms of LA. The only method available to detect LA and DHLA reliably was reported previously from this laboratory using HPLC and electrochemical (EC) detection with dual Au|Hg electrodes.[12] The method was based on the reduction of disulfides in LA at electrode 1 followed by oxidation of thiols at the downstream electrode 2. To avoid oxidation of DHLA to LA during the measurement, oxygen must be excluded rigorously from the system. One of the major drawbacks of the reported assay method is that the Au|Hg electrodes lose sensitivity after a few (30–50) injections and must be reconditioned. This loss of sensitivity of the electrodes is caused mainly by the consumption of Hg at the electrode surface. Reaction of thiols with electrodes slowly depletes the Hg amalgam plating. In addition, the preparation of the Au|Hg amalgam electrode requires cautious handling as is usually recommended for work with mercury. This article reports on an improved, highly sensitive, and more convenient method for the detection of oxidized and reduced LA in biological samples using HPLC-EC coulometric detection.

Principles of HPLC-EC Coulometric Detection

Electrochemistry involves heterogeneous chemical reactions between a compound and an electrode in which an electron is transferred from the solution to the electrode, or vice versa, and a measurable current is formed as a result. For such oxidation–reduction reactions to occur, energy in the

[10] L. Packer, E. H. Witt, and H. J. Tritschler, *Free Radic. Biol. Med.* **19,** 227 (1995).
[11] S. Roy and L. Packer, *Biofactors* **7,** 263 (1998).
[12] D. Han, G. J. Handelman, and L. Packer, *Methods Enzymol.* **251,** 315 (1995).

form of an electric potential is required. In a traditional electrochemical detector, the potential is held constant (DC mode) and the current is measured as a function of time. When an electroactive species flows through the electrode, current is formed. The magnitude of this current is proportional to the concentration of the compound in solution on the electrode. Most electrochemical detectors for HPLC operate in the amperometric mode. In such a mode the solution of the compound only passes over a flatbed of a working electrode. Under such conditions only a small flat surface area of the electrode is available for interaction with the analyte. As a result, only 5–15% of the electroactive species is oxidized or reduced by the electrode. In contrast, coulometric detectors use flow-through or porous graphite electrodes. The surface area of such electrodes is large, allowing almost 100% of the analyte to react with it. Thus, the efficiency in coulometric detection is approximately 100% compared to the conventional amperometric detection that has only 5–15% detection efficiency.

HPLC Apparatus and Analytical Cells

The HPLC system consists of a ESA (ESA Inc., Chelmsford, MA) Model 580 solvent delivery module, a rheodyne injector (Cotati, CA), an on-line degassing system (Altech, Deerfield, IL), and a ESA Coulochem II multielectrode detector (Chelmsford, MA). The detector contains the following analytical cells: (i) a Model 5010 analytical cell (electrode 1), (ii) a Model 5011 high sensitivity analytical cell (electrode 2), and (iii) a Model 5020 guard cell, which is placed between the pump and the injector (ESA Inc., Coulochem II, Chelmsford, MA). The electrodes of the coulometric detector for LA/DHLA and GSH/glutathione disulfide (GSSG) assays are set as follows: electrode 1, 0.40 V; electrode 2, 0.85 V; and guard cell, 0.90 V. Data are collected using a PE Nelson 900 Series Interface and analyzed using software Turbochrom 3 (Perkin Elmer, San Jose, CA).

Column and Mobile Phases

A C_{18} column (150 mm long × 4.6 mm I.D., 5-μm pore size; Alltech, Deerfield, IL) is used for the separation of LA/DHLA and GSH/GSSG. The mobile phase for LA/DHLA separation consists of 50% of 50 m*M* NaH_2PO_4, (pH 2.7), 30% acetonitrile, and 20% methanol. For the separation of GSH/GSSG, the mobile phase consists of 98% 50 m*M* NaH_2PO_4 (pH 2.7) and 2% acetonitrile. For both assays, the flow rate is maintained at 1 ml/min throughout the analysis.

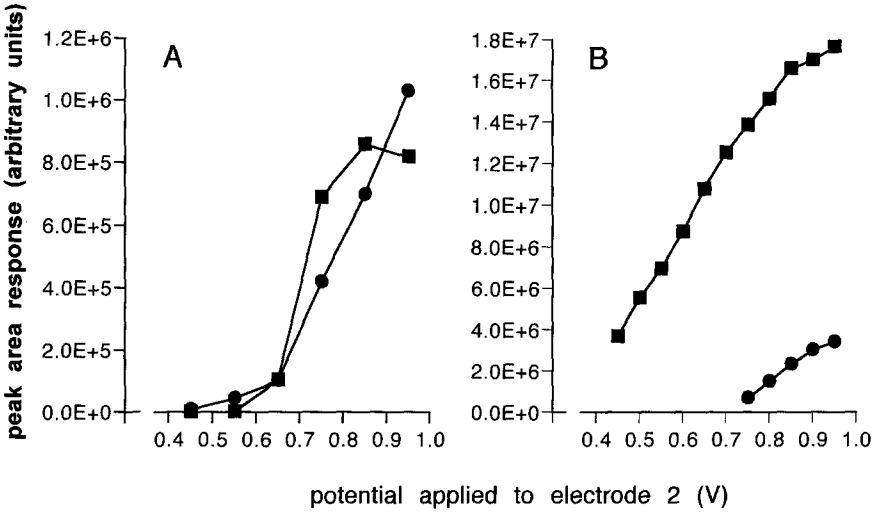

FIG. 1. Current–voltage response curves for lipoate (5 μM LA, ■) and dihydrolipoate (5 μM DHLA, ●) (A) and glutathione (12.5 μM GSH, ■) and glutathione disulfide (5 μM GSSG, ●) (B). As the potential applied to electrode 2 is increased, the peak area response increases for DHLA and GSH. For LA and GSSG the peak area response plateaus at about 0.8 and 0.9 V, respectively.

Current–Voltage Response Curve

The optimal use of an electrochemical detector for liquid chromatography requires knowledge of the appropriate potentials to drive the desired electrochemical reaction. This potential is dependent on a large number of factors, including the nature of the electrode surface, pH, composition of the mobile phase, and chemistry of the compound of interest. A plot of current generated (peak height) versus applied potential difference is commonly referred to as a hydrodynamic (HDV) voltammogram or a current–voltage (C–V) curve. The optimum potential for the oxidation of LA, DHLA, GSH, and GSSG was determined by injecting the compounds onto the column and adjusting the potential difference across electrode 2 from 0.40 to 0.95 V (Fig. 1). The optimum potential for the quantitative measurement of LA/DHLA and GSH/GSSG is 0.85–0.9 V. This range of potential difference allows to obtain maximum peak area with minimal background in response to a given injection of analyte.

Standards and Standard Curve

Lipoate and DHLA standard compounds are from either ASTA-Medica (Frankfurt-am-Main, Germany) or Sigma Chemical Company (St. Louis,

MO). Dihydrolipoate is highly unstable and oxidizes readily at room temperature. To avoid oxidation, DHLA standards are prepared in the mobile phase (pH 2.7) of LA/DHLA and stored at −80° or in liquid nitrogen. Concentrations of LA or DHLA versus the corresponding peak area response are plotted in Fig. 2. The linear range of both compounds is shown as inserts of Fig. 2. Injection of large amounts of the compounds may overwhelm the redox capacity of the electrode and may cause a deviation from the linear relationship between peak area and sample quantity. Repeated regression analysis of standards over a several day period result in high correlations ($R = 0.99$), indicating low variability of analysis in different days. Figure 3 shows standard curves for GSH and GSSG assays. The HPLC-EC coulometric method for the detection of GSH and GSSG has been reported.[13] Concentrations of GSH and GSSG used to prepare the standard curve were kept in a range that matches the concentration of these compounds in biological cell samples (described later).

Detection of LA/DHLA and GSH/GSSG in Biological Samples

To demonstrate that the method is applicable for biological samples, LA/DHLA (Fig. 4) and GSH/GSSG (Fig. 5) were analyzed from human T lymphocyte and rat skeletal muscle-derived L6 cells.

Cells and Cell Culture

Human Jurkat T cells clone E6-1 [American Type Culture Collection (ATCC), Rockville, MD] are grown in RPMI 1640 medium (GIBCO-BRL, Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin/100 μg/ml streptomycin, 110 mg/liter sodium pyruvate, and 2 m*M* L-glutamine (University of California, San Francisco, CA). Rat skeletal muscle-derived L6 myoblasts are also from ATCC. For experiments, cells are seeded at a concentration of 0.5×10^6 cells per well in a 6-well, flat-bottom tissue culture-treated polystyrene plate (Falcon, Becton-Dickinson Labware, NJ). Cultures are grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL) supplemented with 10% FCS, 5 m*M* glutamine, 0.3% D-glucose, 50 U/ml of penicillin, and 50 μg/ml of streptomycin. Cells are maintained in a standard culture incubator with humidified air containing 5% (v/v) CO_2 at 37°.

Extraction of Thiol/Disulfides from Biological Samples

For HPLC analyses, cells are pelleted (400*g*, 5 min) at 4°, and the pellet and medium are deproteinized separately by treatment with 4% (final

[13] J. Lakritz, C. G. Plopper, and A. R. Buckpitt, *Anal. Biochem.* **247,** 63 (1997).

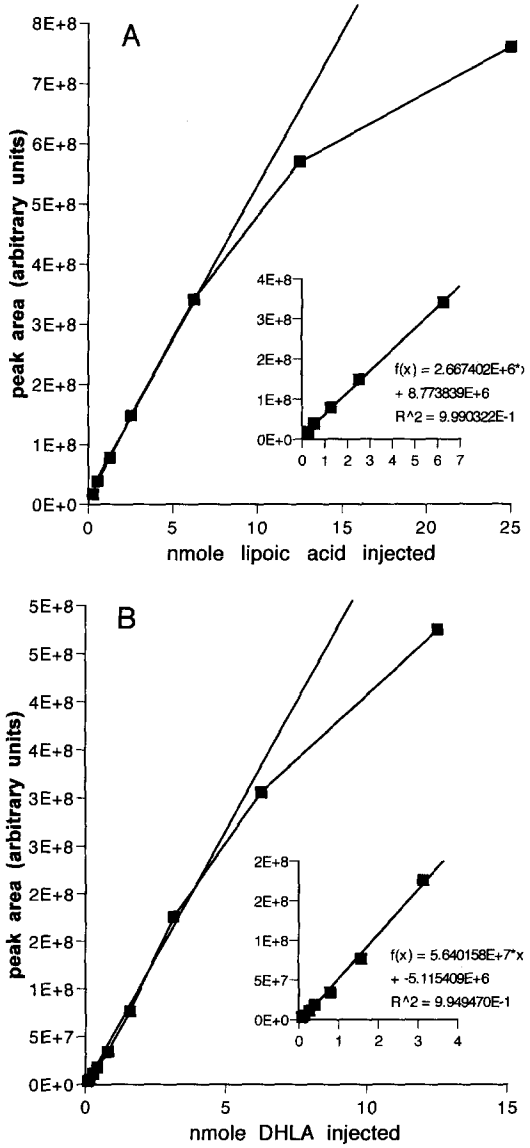

FIG. 2. Representative standard curves for lipoic acid (LA) (A) and dihydrolipoate (DHLA) (B). At high concentrations the peak area response is not linear to the amount of LA or DHLA injected. (Inset) The working range where concentration to the peak area response is linear. The conditions of chromatography are described in the text. Guard cell, 950 mV; screening electrode E1, 400 mV; analytical electrode E2, 850 mV.

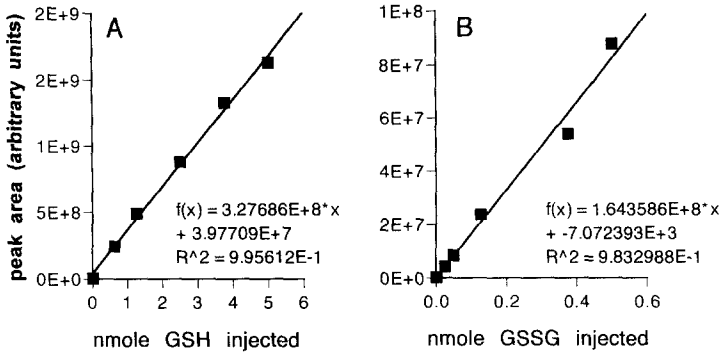

FIG. 3. Representative standard curve for reduced glutathione (GSH) (A) and glutathione disulfide (GSSG) (B). The concentration range plotted for GSH and GSSG was selected to match concentrations that are expected in biological samples. The conditions of chromatography are described in the text. Guard cell, 950 mV; screening electrode E1, 400 mV; analytical electrode E2, 850 mV.

concentration) monochloroacetic acid (Fisher Scientific, Springfield, NJ) for GSH and GSSG measurements. For the measurement of LA and DHLA the cell pellet and supernatant culture medium are deproteinized by treatment with the LA/DHLA HPLC mobile phase, the composition of which is described in a previous section. Following deproteinization, the mixtures

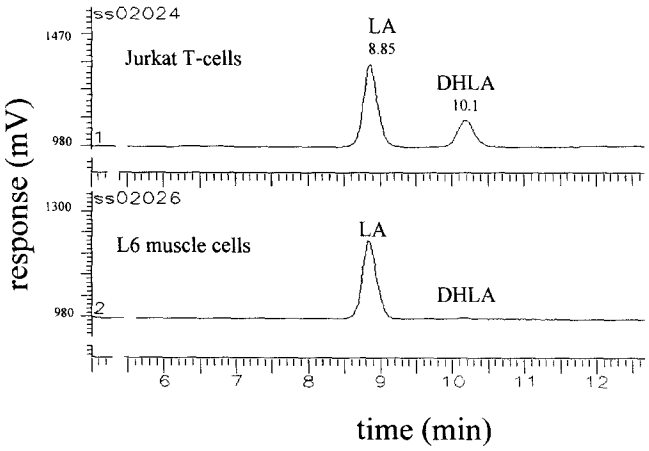

FIG. 4. Reduction of lipoic acid (LA) to dihydrolipoate (DHLA) by human Jurkat T cells grown in suspension culture and rat L6 myoblasts grown in monolayer. Cells were treated with 0.5 m*M* LA for 30 min. LA and DHLA were detected in cells using the HPLC coulometric detector as described in the text. Individual LA and DHLA peaks in each chromatogram are labeled.

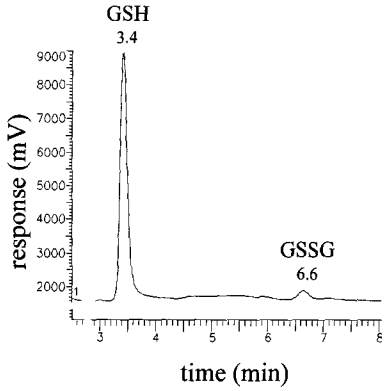

FIG. 5. Chromatogram of glutathione (GSH) and glutathione disulfide (GSSG) detected in a Jurkat T-cell extract. Following acid extraction of cells, GSH and GSSG were detected using the HPLC coulometric detector as described in the text. Individual GSH and GSSG peaks are labeled.

are snap-frozen in liquid nitrogen and stored at −80° for HPLC determinations. The HPLC assay is done within 1 week of storage. Immediately before the assay, samples are thawed, vortexed, and then centrifuged at 15,000*g* for 2 min. The clear supernatant is filtered using microfilterfuge tubes (Rainin, Woburn, MA) fitted with a 0.45-μm nylon filter and used for injection.

Cellular Reduction of LA to DHLA

Exogenous free LA added to Jurkat T cells in culture is taken up rapidly by the cells and reduced to DHLA.[12,14] Figure 4 illustrates the presence of DHLA in human Jurkat T cells and traces of it in L6 skeletal muscle cells that were treated with 0.5 m*M* of LA for 30 min. No DHLA is detected in the cells immediately after the addition of LA (not shown), suggesting that the DHLA detected after 30 min of treatment is formed in the cells. The reduction of LA to DHLA is more marked in Jurkat T cells compared to that in L6 cells where only trace amounts of DHLA could be detected.

In summary, the HPLC-EC coulometric detection method described here sensitively detects both oxidized and reduced species of lipoate (Fig. 4) and glutathione (Fig. 5) from biological samples. Detection limits for LA and DHLA for this HPLC method are 1–5 pmol, which is at least 10 times more sensitive for the detection of LA compared to the Au|Hg electrode-based detection reported previously.

[14] G. J. Handelman, D. Han, H. Tritschler, and L. Packer, *Biochem. Pharmacol.* **47,** 1725 (1994).