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Rapid Analysis of Glutathione in Human Prostate Cancer Cells (DU145) and Human Lung Adenocarcinoma Cells (A549) by HPLC with Electrochemical Detection

A. Spadaro*, G. Ronsisvalle and Maria Pappalardo

Dipartimento di Scienze del Farmaco, Università di Catania, Viale Andrea Doria 6, 95125 Catania, Italy
*E-Mail: angelo.spadaro@unict.it

Abstract
Here we provide an extremely simple, rapid, direct, sensitive and specific method to quantify for the first time intracellular GSH in human prostate cancer cells (DU145) and human lung adenocarcinoma cells (A549) using an electrochemical detector coupled to a high performance liquid chromatograph (HPLC-ECD). GSH determination in cancer cells are importance because GSH has been involved in the development of resistance to several anticancer drugs. Separation of GSH was performed on a Alltima C18 column using a binary pre-mixed mobile phase of methanol-NaH_2PO_4 (10 mM, pH 3.0 with phosphoric acid) (2:98 v/v). GSH was detected with an oxidation potential of + 500 mVolt. The limit of detections was 0.33 pMol. Linearity, accuracy and precision were found to be acceptable. The experiments show that the HPLC-ECD method developed in this study could represent an useful alternative to the existing procedures since its simplicity and rapidity.

Key Words
Glutathione, HPLC, electrochemical detection, DU145, A549, cell cultures.

INTRODUCTION

Glutathione (GSH) occur widely in living tissues and represents the most abundant low molecular mass thiol found in cells. GSH plays an important role in human metabolism, including the detoxification of xenobiotics, cell homeostasis, radioprotection and antioxidant defense [1]. Depletion of GSH is associated with a variety of human diseases including diabetes, AIDS and neurodegenerative diseases [2-4].

Human prostate cancer cells (DU145) and human lung adenocarcinoma cells (A549) are two cancer cell lines extensively used in cancer research. GSH determination in cancer cells, such as DU145 and A549 cells, is of particular interest, i.e. GSH and glutathione-*S*-transferase (GST) have been implicated in the development of resistance to several anticancer drugs. As is well known, electrophilic drugs may be catalytically conjugated to GSH by GST and the resultant thioether product effluxed from cells by the multidrug resistance-associated protein 1 (MRP1) [5-7].

Various analytical methods, such as enzymatic [8,9] and fluorimetric [10] assays, have been developed for determination of GSH. To improve the specificity and sensitivity several HPLC methods for the determination of GSH using different detection systems, as UV [11], fluorometer [12, 13], electrochemical detector (ECD) [14-18] and mass spectrometry [19] were developed.

To date, the intracellular GSH level, in DU145 and A549 cell lines, is carried out spectrophotometric or fluorescence methods [20, 21] and no HPLC method with electrochemical detection were reported. Recently, it has been demonstrated that in the spectroscopic assays of GSH other thiols are wrongly detected [22].

In this study, we provided a simple, rapid, direct, sensitive and very specific reverse-phase HPLC-ECD method with isocratic elution to detect GSH in vitro cultured DU145 and A549 cells. The proposed HPLC method is extremely rapid and straightforward since has a very short sample preparation procedure and does not require chemical derivatization of GSH.

Furthermore, the applicability of this procedure to the assay of GSH in these cell lines was investigated as well as several factors that might influence the final results of the method.

MATERIALS AND METHODS

Chemicals

GSH was obtained from Sigma Chemical Co. (Milano, Italy). Water and methanol (HPLC grade) were from Merck (Milano, Italy). The reagents for the bicinchoninic acid (BCA) protein assay were

from Pierce (Celbio, Pero, Italy). All other chemicals were reagent grade.

HPLC Apparatus

The HPLC apparatus consisted of two Model 510 pumps, a Model 712 Wisp auto-injector and an electrochemical detector (Model 5100A Coulochem, ESA, Bedford, MA, USA) which consisted of a control module and an analytical cell (Model 5011A Coulochem, ESA, Bedford, MA, USA) containing two on-line porous graphite coulometric electrodes. The analysis was performed in the oxidative mode with a potential of + 500 mVolt. A guard cell (Model 5020, Coulochem ESA, Bedford, MA, USA) held at + 900 mVolt, placed between the pump and the injector, was used to minimize the background current. Signals from the detectors were elaborated an APC IV computer system (NEC, Boxborough, MA, USA) using Maxima 820 software (Waters Assoc., Milford, MA, USA). The mobile phase was filtered through a GS-type filter (0.22 mm,Millipore, Bedford, MA, USA) and degassed on-line with a model ERC-3311 solvent degasser (Erma, Tokyo, Japan).

Chromatography Conditions

Separations of GSH were performed on a Alltima C18 5µm (25 cm x 4.6 mm) (Alltech, Milano, Italy) using a binary pre-mixed mobile phase of methanol-NaH_2PO_4 (10 mM, pH 3.0 with phosphoric acid) (2:98 v/v) at a flow rate of 1.0 mL/min with an injection volume of 20 µL. Chromatography was performed at room temperature (21 °C) and external standardization was used.

Optimization of electrochemical detection

A hydrodynamic voltammogram, in the range 0-900 mVolt, was generated by repeated injections of GSH standard to assess the optimum potential for oxidative coulometric detection.

Standard Solutions

Standard solutions of GSH in trichloroacetic acid (TCA) 0.5% in the concentration range 1-200 nM and 1-300 ng/ml were prepared by diluting known amounts of stock solutions, containing 100 µM/ml of the thiol and immediately analyzed by HPLC-ECD.

Cell cultures

DU145 and A549 cells obtained from American Type Culture Collection (American Type Culture Collection (ATCC), Manassas, VA, USA) were cultured by normal growth conditions as previously reported [23, 24].

Sample preparation

DU145 and A549 cells monolayers, rinsed with PBS (+4°C), were scraped and centrifuged at 800 x g for 10 min at +4°C. The pellets were treated with 400 µL of 5% TCA at 4°C and centrifuged at 2000 x g for 10 min at 4°C. The acidic TCA solutions were opportunely diluted and injected into the HPLC column. GSH is relatively stable in acidic solution, but it is recommended that the samples are kept on ice throughout the preparation. The GSH content was expressed nMol/mg of proteins. Protein content was evaluated in the pellets by the bicinchoninic acid (BCA) protein assay [25].

Statistical analysis

Results are expressed as means±SD.

RESULTS AND DISCUSSION

Analysis of GSH in DU148 and A549 cell samples

In this study, we provided a reverse-phase HPLC-ECD method to detect intracellular GSH in DU145 and A549, two cell lines very used in cancer research. The advantages of this chromatographic method over existing methods are: fast chromatographic run, very short sample preparation, no derivatization procedures, and the use of selective detection to eliminate interference from biological matrices.

Until now, the intracellular GSH determination, in these cells line, is carried out spectrophotometric or fluorescence methods [20, 21]. Recently, it has been demonstrated that in the spectroscopic assays of GSH, other thiols, including protein-bound thiols, are falsely detected with 1.3-2.1-fold increase respect to chromatographic assay [22].

For the determination of the optimum parameters for the electrochemical detection of GSH the voltammogram curve were determined (Fig. 1). A response due to the oxidation of the thiol group of GSH was observed for a potential of greater than 200 mVolt. In accordance with previous work, GSH showed a further voltage-dependent increase before reaching the final plateau due to the oxidation of the amine group(s) [17]. We decided as working potential +500 mVolt because in our conditions this value correspond to the better compromise between sensitivity and selectivity. At this value we was able to detected about 50% of the overall GSH signal, with reasonable sensitivity.

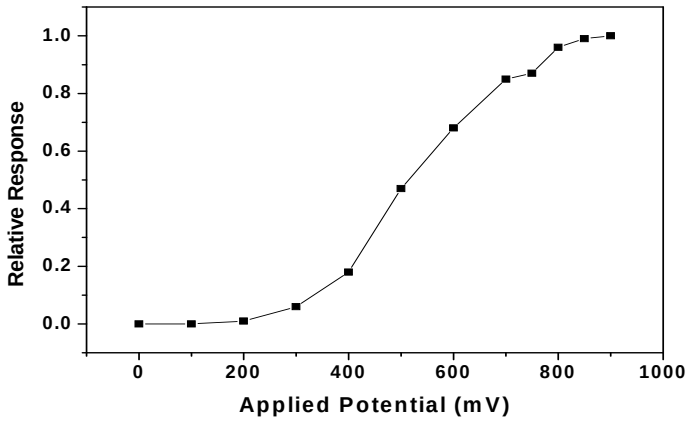

Fig. 1. Hydrodynamic voltammogram of GSH.

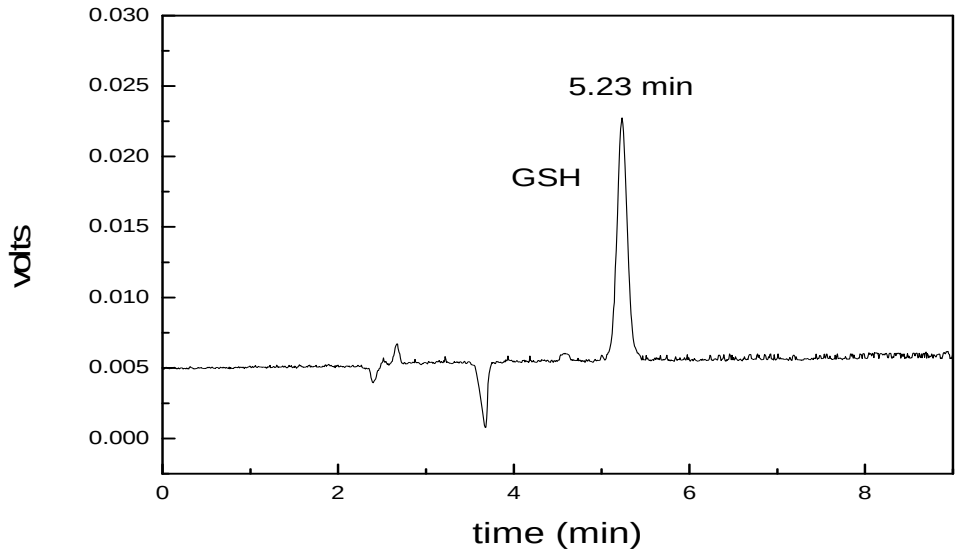

Fig. 2. Representative chromatogram of glutathione (GSH) in a cell sample.

Moreover, the enhanced selectivity obtained removes the need of complete resolution of the GSH from endogenous compounds present in the DU145 and A549 cell homogenates avoiding any time-consuming sample preparation. Therefore, the method described constitutes an useful approach for fast screening and routine analysis of GSH in DU145 and A549 cells, two cellular lines commonly used in cancer research.

Chromatographic separations were carried out under reverse phase conditions on a Alltima C18, 5µm column using a binary eluent, MeOH/phosphate-buffer at acidic pH. The isocratic conditions described provided good chromatographic peak shape with a complete and rapid baseline resolution of the GSH and a relatively short analysis time. GSH eluted at 5.23 min, although the total analysis lasted 9 min to provide thorough washing of the column between injections. Therefore this chromatographic system was adopted in the cell analysis studies. Fig. 2 shows typical chromatogram obtained by cell samples whereas the GSH contents are reported in Table 1.

Table 1-Concentrations of glutathione (GSH) in Human Prostate Cancer Cells (DU145) and Human Lung Adenocarcinoma Cells (A549)

	GSH (nMol/mg protein)*
DU145	20.21±2.44
A549	30.11±1.53

*Values indicate nMol of GSH per mg of proteins (mean±SD of 6 experiments)

Table 2 Intra-day accuracy and precision in the analysis of GSH in DU145 and A549 cells

True conc. (nMol/mg protein)	Accuracy[a] Mean±SD		RSD[b] (%)	
	DU145	A549	DU145	A549
1	97.8±2.16	97.10±2.41	2.21	2.48
10	98.91±1.56	98.21±1.74	1.58	1.77
100	100.90±1.11	101.80±1.33	1.10	1.31

[a,b] n=6

Table 3 Inter-day accuracy and precision in the analysis of GSH in DU145 and A549 cells

True conc. (nMol/mg protein)	Accuracy[a] Mean±SD		RSD[b] (%)	
	DU145	A549	DU145	A549
1	98.21±2.44	98.60±2.31	2.48	2.34
10	98.80 ±2.41	98.87±2.22	2.44	2.24
100	102.11±1.53	99.99±1.77	1.50	1.77

[a,b] n=6

Sample preparation

The major weak points in the GSH determination are the easy GSH autoxidation, and the enzymatic proteolysis of GSH by γ-Glutamyl-Transferase (γ-GT). GSH rapidly oxidizes non-enzymatically at pH values greater than 7, and γ-GT, which catalyzes the first step of GSH proteolysis, has an optimum of activity at neutral pH [26, 27]. Thus, it is suggested to maintain the pH of media where GSH is processed below pH 7 [28]. For these reasons during sample preparation, cell samples were homogenized in 5% TCA to prevent auto-oxidation of thiols and thiol–disulfide exchange.

Calibration and linearity

An external standard method was used for quantitative determinations of GSH in DU145 and A549 cells. Linearity was evaluated in the range 1-300 ng/ml by analysis of standard solutions of GSH. The peak area ratio and concentration was subjected to regression analysis to calculate the calibration equations and correlation coefficients. The regression equation obtained was $Y = 3.72654 \cdot 10^{-5} \cdot X + 5.96804$ (r=0.9986, n=6). The results show that within the concentration range indicated there was an excellent correlation between peak area ratio and concentration of GSH.

Determination of the limit of detection and quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) of the present method were determined in cell homogenate samples of both cell lines spiked with progressively lower concentrations of GSH. LOD was found to be 0.33 pMol for both DU145 and A549 cells with a signal/noise ratio of about 3:1 (n=6). LOQ was found to be 0.92 pMol for both cell lines with a signal/noise of about 10:1 (n=6).

Accuracy and Precision

The precision and accuracy of the extraction procedure and chromatography were investigated at three concentrations levels in DU145 and A549 cell homogenates. For both cellular lines the homogenates were split into two aliquots. The first aliquot was further divided in three parts and spiked separately of three levels of known amount GSH (1, 10 and 100 nMol/mg protein). The second homogenate aliquot, divided in two parts, was used to determine the protein content and the basal levels of the GSH that was subtracted from the values founded in the first aliquot.

The accuracies of the assays were determined by comparing the measured concentration to its true value. The precision of the method was evaluated by replicate analysis and was expressed as RSD. The intra-day precision and accuracy were checked by replicate analysis on the same day. The inter-day precision and accuracy were evaluated on five consecutive days on fresh cell homogenates.

Tables 2 and 3 showed that the assay was highly reproducible with low intra- and inter-day variation. In fact, the intra-day and the inter-day recoveries range from 97.10 to 101.80% and from 98.21 to 102.11% respectively, whereas the accuracies were less than 2.44% in both cases.

CONCLUSIONS

The analytical methods reported in this study permit for the first time the determination of GSH by HPLC coupled with ECD in DU145 and A549 cell lines. The experiments show that the method developed has good repeatability, selectivity and stability in detecting GSH in the cell lines studied. The linear ranges cover over two orders of magnitude and the limits of detection is in the pMol range. Therefore, this analytical method can be used where limited quantities of samples are available, and consequently is suitable for the analysis of GSH in DU145 and A549 cells to study biochemical phenomena in which the GSH is involved, i.e. in the anticancer resistance mechanisms. The proposed method could represent an useful alternative to the existing procedures since its simplicity and rapidity.

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