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Short communication

Determination of glutathione in single human hepatocarcinoma cells by capillary electrophoresis with electrochemical detection

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Abstract

A method for determination of glutathione (GSH) in single human hepatocarcinoma (HH) cells was described by capillary zone electrophoresis with electrochemical detection at a gold/mercury amalgam micro-disk electrode. When HH cells were washed with the running buffer instead of physiological buffer saline, only one electrophoretic peak for GSH is depicted on the electropherograms of single HH cells. When electroosmotic injection of 0.01 mol/l NaOH for lysing the cell introduced into the capillary, the lysis time can be shorten to 5 s. The whole cell injection and no need of derivatization reaction lead more accurate and precise results. The average amount of GSH in an individual HH cell is 22.3±5.8 fmol (mean±standard deviation), which is consistent with that of its homogenate.
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1. Introduction

Glutathione (L-γ-glutamyl-cysteinylglycine, GSH) is the most abundant low-molecular mass thiol in mammalian [1–3]. It is important for protein preservation in most living cells and participates in the reduction of disulfides and other molecules, and conjugates with compounds of exogenous and endogenous and free radicals [4]. The amount of GSH in tumor cell lines is usually much higher than that in normal cells [5,6]. Therefore, determination of GSH in human hepatocarcinoma (HH) cells is an important and significant work. High-performance liquid chromatography with electrochemical detector, in which GSH was derivatized with an electroactive reagent, has been used for determination of GSH in tumor cells [7].

In routine tests, hundreds of thousands of cells are homogenized to provide a sufficient amount of analytes for quantification. The results obtained cannot reflect the real situation of each cell. Single-cell analysis can potentially benefit to obtain the information of individual cells. Capillary electrophoresis (CE) has established itself as a powerful micro-scale separation tool [8]. The ability of CE to extremely small sample volumes and to selectively determine multiple analytes using the technique's high efficiency and resolution has led to the application of CE to the analysis of the chemical content of single cells [9]. Capillary electrophoresis with laser-induced fluorescence (LIF) detection has been used

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for determination of GSH in single human erythrocytes [10,11], in which GSH has to be derivatized with a fluorescent reagent. Incompleteness and nonuniformity in the extent of the derivatization reaction lead to deviation of analytical results [10]. In previous papers, we provided the methods for determination of GSH in single human erythrocytes [12] and single mouse peritoneal macrophages [13] by capillary zone electrophoresis (CZE) with electrochemical detection. No derivatization is needed in the scheme. However, a large electrophoretic peak corresponding to the physiological buffer saline (PBS), in which the cells are present, appears. Both peaks of PBS and GSH must be separated before detection. In addition, the lysis of the single macrophage in the capillary is difficult with the running buffer. When 0.01 mol/l NaOH is introduced by diffusion into the capillary around the cell, lysis of the cell takes ca. 120 s. The long lysis time will dilute the analytes in the cell. In this work, we improved the method. No separation is needed, because only one electrophoretic peak corresponding to GSH is depicted on the electropherograms of single HH cells. The lysis period is shortened to 5 s.

2. Experimental

2.1. Apparatus

The CZE/electrochemical detection system used in this work was similar to the one described previously [14]. A high-voltage power supply (Model NT-9123, Beijing Institute of New Technology, Beijing, China) provided a variable voltage of 0–30 kV across the separation capillary with the outlet of the capillary at ground potential. Fused-silica capillaries (20 μm I.D., 375 μm O.D.) were purchased from Yongnian Optical Conductive Fiber Plant, Yongnian, China. The uncoated capillaries could be used directly. The electrochemical detection at a constant potential of 0.20 V was performed using an electrochemical analyzer (Model CHI 802, CH Instrument, Austin, TX, USA). The home-made detection cell (ca. 250 μl) was housed in a Faraday cage in order to minimize the interference from noise of external sources. Electrochemical detection was carried out with a three-electrode system. It consisted of a gold/mercury amalgam micro-disc electrode as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and a coiled Pt wire (0.3-mm diameter, 5-cm long) as the auxiliary electrode. The gold/mercury amalgam micro-disc electrodes were similar to our previous description [15].

2.2. Reagents

GSH (content >98%) was obtained from Acros Organic (NJ, USA). A 1.00×10^{-2} mol/l stock solution of GSH was prepared in 2.0×10^{-2} mol/l Na_2H_2EDTA. Other reagents were of analytic grade, purchased from standard suppliers. PBS consisted of 0.135 mol/l NaCl and 0.02 mol/l NaH_2PO_4 and was adjusted to pH 7.4 with 0.1 mol/l NaOH. The electrophoresis buffer was 1.22×10^{-3} mol/l Na_2HPO_4–7.80×10^{-4} mol/l NaH_2PO_4–2.0×10^{-3} mol/l Na_2H_2EDTA (pH 7.0). All solutions were prepared with double-distilled water.

2.3. Procedure

2.3.1. Preparation of cell and its extract

HH cells with a diameter of about 24 μm were provided by School of Medicine, Shandong University. HH cells suspended in approximately 3-ml culture medium were centrifuged for 10 min at 1000 rpm and the supernatant was discarded. In order to remove the residual culture medium, ca. 1 ml running buffer was added. After the cell mixture was blown gently with a pipette, it was centrifuged for 10 min at 1000 rpm. The supernatant was discarded again. The step was repeated twice. Then the cells were suspended in the running buffer of 1 ml. This was the cell suspension for single-cell analysis. The amount of HH cells in the running buffer was counted using a hemocytometer (Shanghai Medical Optical Instrument Plant, Shanghai, China). Yield was 6.35×10^5 cells/ml. In order to obtain the extract of HH cell, 0.2 mol/l NaOH of 10 μl was added into the cell suspension of 200 μl and the mixture was sonicated for 5 min.

2.3.2. Electrophoresis

Before use, the capillary was flushed with 0.2 mol/l NaOH, double-distilled water and the running buffer for 5 min, respectively. The working electrode was aligned to the capillary outlet was

carried out in horizontal direction by adjusting a home-made three-dimensional micro-manipulator under a stereo microscope. With the aid of a small normal mirror, the vertical alignment was carried out using the micro-manipulator. Then a voltage of 20.0 kV was applied across the capillary and the detection potential was applied at the working electrode. When the electroosmotic flow reached a constant value, the electromigration injection of the sample solution or the extract of HH cell was performed at 5.0 kV for 10 s. After the capillary was put back to the running buffer, the separation voltage was applied again and the electropherogram was recorded.

2.3.3. Single cell analysis

A droplet of the HH cell suspension of 50 μl was placed on a clean microscope slide. Then the microscope slide was placed on the inverted biological microscope with a magnification of ×600, the injection end of the capillary filled with the running buffer was gently immersed in the droplet under the guidance of a three-dimensional micro-manipulator. In order to see the opening of the injection end clearly, the polyimide-coating of ca. 5 mm at the injection end of the capillary was removed by burning before use. A platinum wire was placed in the cell suspension to serve as the electrophoresis anode. As soon as one HH cell was drifting towards the injection end, an injection voltage of 5.0 kV was applied to transport the whole cell into the capillary tip. When the cell was injected into the inlet of capillary, the high voltage was turned off. The cell suspension on the microscope slide was replaced by 0.01 mol/l NaOH of 50 μl and a voltage of 5.0 kV was added across the capillary for 5 s. Then the injection end of the capillary was put to the reservoir containing the running buffer. Finally, the separation voltage was applied and the electropherogram was recorded.

3. Results and discussion

3.1. Determination of GSH in the extract of HH cell

The experimental conditions of detecting GSH by CZE were 1.22×10^{-3} mol/l Na_2HPO_4–7.8×10^{-4} mol/l NaH_2PO_4–2.0×10^{-3} mol/l Na_2H_2EDTA (pH 7.0) for the running buffer, 20.0 kV for the separation voltage, 0.20 V versus SCE for the detection potential, 5 kV for the injection voltage and 10 s for the injection time. The response for a series of six injections of 5.00×10^{-5} mol/l standard GSH resulted in a relative standard deviation of 3.2% for the migration time and 5.2% for the electrophoretic peak area, respectively. The limit of detection was 1.7×10^{-6} mol/l or 2.2 fmol (according to ratio of signal to noise of 3) for the injection volume calculated. A linear relationship held between the peak area detected and concentration in the range of 2.00×10^{-6} to 5.00×10^{-4} mol/l. Least-squares treatment of these date yielded a slope of 4.65 μC mol^{-1} l and a correlation coefficient of 0.999.

Under these conditions, the peak corresponding to GSH, eluting at 4.52 min, appeared on the electropherogram of the extract of HH cell. In order to quantify the concentration of GSH in the extract, the calibration curve was used. The concentration of GSH was determined to be 16.2±2.4 μmol/l (mean±standard deviation, $n=5$). The recovery of the method for GSH in the extract was between 96 and 105%. Since the cell concentration in the extract was 6.35×10^{5} cell/ml, the mean amount of GSH in a single HH cell could be calculated to be 25.5±3.8 fmol.

3.2. Single-cell analysis

For single-cell analysis using CE-LIF detection reported in literature, PBS, in which the cells are present, cannot interfere with the determination of GSH because there is no fluorescence signal for PBS [10,11]. However, Cl^- in PBS produces an electrophoretic peak for electrochemical detection, because it can be oxidized at the gold/mercury amalgam electrode. The peak of PBS must be separated from the peak of GSH. It was found that if PBS was replaced by the running buffer, HH cells can be living over 2 h and only one peak for GSH depicts on the electropherogram of a HH cell. Therefore, the running buffer is selected to wash and suspend HH cells. Fig. 1 shows the electropherograms of a standard solution of GSH and a HH cell.

After injecting a cell into the capillary, the cell must be lysed. The lysis process must preserve the integrity of the relevant compounds at their pre-lysis conditions while releasing them from the cell. Usual-

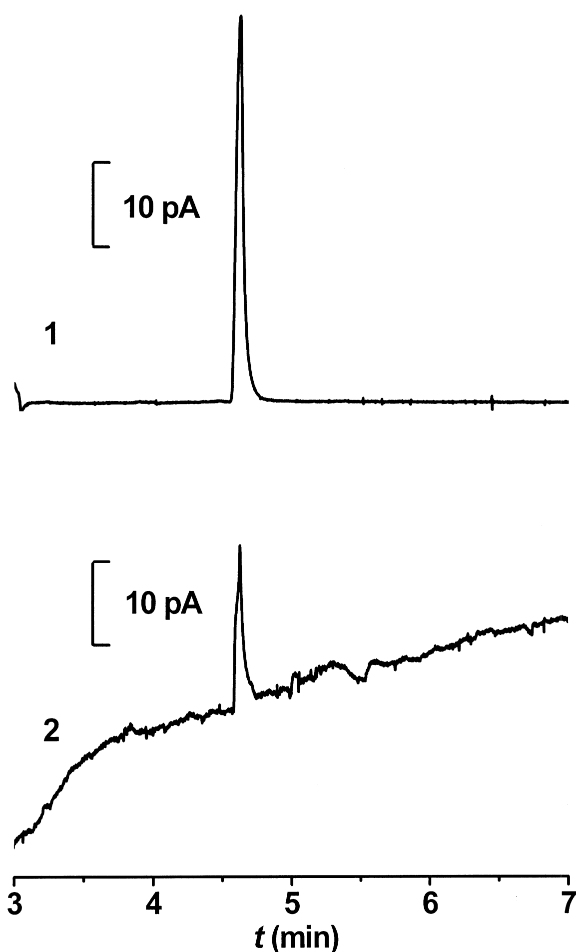

Fig. 1. Electropherogram of (1) 5.00×10^{-5} mol/l GSH, injection, 5.0 kV for 10 s and (2) a HH cell. Running buffer, 1.22×10^{-2} mol/l Na_2HPO_4–7.80×10^{-3} mol/l NaH_2PO_4–2.0×10^{-3} mol/l Na_2H_2EDTA, pH 7.0; capillary, 45 cm×20 μm I.D.; separation voltage, 20.0 kV; detection potential, 0.20 V (versus SCE).

ly, chemical lysis is accomplished. Erythrocytes can be lysed easily in the running buffer, pH 7.0 [12], but HH cells cannot. We find that HH cells can be lysed in 0.01 mol/l NaOH. If a 5.0-kV high voltage is applied across the capillary to inject 0.01 mol/l NaOH, the lysis time is shorten to 5 s, much shorter than 120 s described previously [13]. The short lysis time effectively minimizes dilution of GSH released from the single cell during the lysis.

Due to the small volumes and low analyte levels, the internal standard method of individual cells was difficult to analyze [10]. The reproducible peak area together with the large linear dynamic range for standard GSH made it suitable to use calibration curve for the quantification of GSH in single HH cells. The results of consecutive analysis of seven HH cells were listed in Table 1. There is a clear drift in migration time. Overall ζ-potential change, resulting from the adsorption of the substances in the cells on the inner surface of the capillary wall, is probably responsible for this. There is no correlation between the peak area and the run number, which proves that the other substances in the cells do not interfere with the determination of GSH in single HH cells. The average amount of GSH in seven HH cell is 22.3±5.8 fmol (mean±standard deviation), which is consistent with the value obtained by determining the extract of HH cell (25.5±3.8 fmol).

Table 1
Migration time and peak area of the electrophoretic peak, and amount of GSH in single HH cells

Run no.	Migration time (min)	Peak area (nC)	Amount (fmol/cell)
1	4.52	60.8	26.4
2	4.67	67.8	29.4
3	4.72	50.4	21.9
4	4.85	47.8	20.8
5	4.87	41.2	17.9
6	5.07	61.6	26.7
7	5.15	29.5	12.8

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