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Talanta 65 (2005) 986–990
Talanta
www.elsevier.com/locate/talanta

Quantification of glutathione and glutathione disulfide in human plasma and tobacco leaves by capillary electrophoresis with laser-induced fluorescence detection

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Received 18 April 2004; received in revised form 22 August 2004; accepted 24 August 2004
Available online 30 September 2004

Abstract

A new capillary electrophoresis (CE) method with laser-induced fluorescence (LIF) detection was developed for the rapid separation and sensitive detection of glutathione (GSH) and glutathione disulfide (GSSH) after derivatization by 4-chloro-7-nitrobenzo-2-oxa-1,3-diazol (NBD-Cl). The derivatization and separation conditions were investigated in detail and the optimums were obtained. Under the optimum experiment conditions, linear relationships between the peak height and concentrations of the analytes in normal and second-derivative electrophoregrams were obtained (0.22–45.00 μM). The detection limits for glutathione and glutathione disulfide in normal and second-derivative electrophoregrams were 0.046 and 0.012 μM and 0.046 and 0.014 μM, respectively. The method was applied to the analysis of glutathione and glutathione disulfide in human plasma and tobacco leaves with satisfactory results.

Keywords: Glutathione; Glutathione disulfide; Capillary electrophoresis; Laser-induced fluorescence

1. Introduction

Glutathione (γ-Glu-Cys-Gly, GSH) is one of the most abundant low-molecular weight thiol-containing species in mammalian tissues [1,2]. It is often involved in protective and detoxifying functions of the cell, such as maintaining the proper thiol-disulfide status of proteins and quenching reactive free-radical species. GSH also participates in other important biochemical reactions including DNA [3], protein synthesis and amino acid uptake [1,2]. Also, GSH protects against excitotoxin-induced brain damage [4] and may be involved in HIV expressions [5]; pro-GSH drugs have been proposed for anti-HIV therapy [6,7]. Some cases of drug and radiation resistance in cancer therapy have been attributed to abnormally high concentration of GSH in tumor cells [8]. On the other hand, glutathione disulfide (GSSG) is known to be formed in biological systems because of the function of GSH as antioxidant [9]. Nowadays, the concentration levels of GSH and GSSG are used for ascertaining the redox status in biological systems and the conversion of GSH to GSSG is widely recognized as a reliable index of oxidative stress [10].

Analysis of GSH and GSSG is of continuous interest because of their biological and clinical significance. High-performance liquid chromatography (HPLC) is used mainly for the analysis of GSH and/or GSSG [11]. However, HPLC methods are limited by long separation time and tedious sample pretreatment steps. Owing to the high separation power, short analysis time and lower operating cost, capillary electrophoresis (CE) has also been used to analyze GSH and/or GSSG in various samples, but the use of CE with UV and electrochemistry detector only offered sensitivity in μM range. He et al. [12] reported a CE-MS method for the assay of GSH in urine samples, but the limit of detection is still high ($0.08\ mg\ ml^{-1}$). Recently, CE methods with laser-induced fluorescence (LIF) detection have been applied to the assay of GSH with high sensitivity [11,13–17], but GSSG is scarcely concerned. Also, these methods have a long separation time (> 8 min generally) or are limited by derivatization selectivity

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doi:10.1016/j.talanta.2004.08.030

[14]. Furthermore, none of the existing CE-LIF methods has aimed at the analysis of GSH and GSSG in plants.

In this paper, we presented the development of a rapid and sensitive CE-LIF method for the simultaneous determination of GSH and GSSG in human plasma and plants. In this work, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazol (NBD-Cl) [18–21] was chosen for the derivatization of the two compounds due to (1) its good matching with our commercial LIF instrument (excitation 488 nm/emission 520 nm), (2) short derivatization time and (3) the stability of amine-derivatives at room temperature. The derivatization and separation conditions were investigated to obtain the optimums and the established method was validated with analyzing GSH and GSSG in human plasma and tobacco leaves.

2. Experimental

2.1. Apparatus

All separations were performed on a P/ACE 5510 system (Beckman Coulter Instrument, Fullerton, CA, USA) equipped with an LIF detector. The excitation light from an argon ion laser (3 mW) was focused on the capillary window by means of a fiber-optic connection. The excitation was performed at 488 nm and a 520 nm band-pass filter was used for emission. The system was controlled by P/ACE^{TM} Station software. The separation was carried out on a 47 cm (40 cm to the detector) × 75 μm i.d. fused-silica capillary (Yongnian Photoconductive Fiber Factory, Hebei, China). The capillary was treated prior to its first use by flushing with 1.0 M HCl for 20 min, 0.5 M NaOH for 20 min and distilled water for 10 min. Between two runs, a rinse-cycle—0.5 M NaOH for 1 min, distilled water for 1 min and run buffer for 2 min—was used. The capillary was maintained at 25 °C. Sample was injected by applying a pressure of 0.5 psi for 5 s.

2.2. Materials

Standard GSH and GSSG were obtained from the Chemical Reagent Service Station of Shanghai. NBD-Cl was the product of Tokyo Kasei Kogyo Co., Ltd (Japan). Plasma sample of a kidney-replant patient was obtained from the Chief Hospital of Army (Gansu, China). The dry tobacco leaves were obtained from a local farm. All other reagents were of analytical reagent grade. Stock solutions of GSH and GSSG at 0.9 mM were prepared in distilled water and a 16.2 mM stock solution of NBD-Cl was prepared in acetonitrile. All the stock solutions were stored at 4 °C.

2.3. Preparation of the electrolytes

The derivatization buffers were prepared from 100 mM borate solution and organic solvent (methanol, ethanol and acetonitrile). The run buffers were prepared by mixing 100 mM borate and 20 mM β-CD solutions. All the buffers were adjusted to the desired pH with 1.0 M HCl or 0.5 M NaOH.

2.4. Sample preparation

2.4.1. Human plasma sample

0.2 ml human plasma of the patient was vortex-mixed with 0.4 ml acetonitrile for 3 min and made to stand for 15 min. Then the resulting solution was centrifuged at 4000 rpm for 5 min to separate the precipitated proteins. The supernatant was derivatized for determination.

2.4.2. Dry tobacco leaf sample

The dry tobacco leaves were pieced and ground. Then, 0.20000 g powder was weighed accurately and extracted with 5.0 ml distilled water in an ultrasonic bath for 1.5 h. After it was filtered with filter paper and 0.45 μm membrane filter, the resulting solutions were derivatized directly.

2.5. Derivatization procedure

In 1.5 ml-plastic tubes, 25 μl standard solutions of GSH and GSSG were mixed with 200 μl NBD-Cl solution and the derivatization buffer. For samples, 100 μl human plasma sample solution and 300 μl tobacco leaf sample solution were mixed with 200 μl NBD-Cl solution and derivatization buffer, respectively. Blank solutions were prepared by mixing 200 μl NBD-Cl solution and derivatization buffer. All mixtures were diluted to 1.0 ml with distilled water and kept in a hot water bath to react. Prior to analysis, the derivatization solutions were diluted with distilled water to the desired concentrations.

3. Results and discussions

3.1. Derivatization and separation of the analytes

Derivatization of GSH and GSSG with NBD-Cl was investigated to achieve higher sensitivity. The effects of predominant parameters, such as reaction time, reaction temperature, buffer pH, buffer concentration and organic solvents, on the fluorescence intensity were investigated. The results indicated that addition of organic solvents (methanol and ethanol) hardly affect the fluorescent intensity of the analytes. Finally, the derivatization conditions were selected as 20 min, 50 °C, pH 8.75 and 20 mM borate buffer.

The parameters that affect the separation were also tested in detail. The experiment results showed that the analytes can be separated better under conditions of pH, 9.25; 20 mM borate buffer; separation temperature, 25 °C; separation voltage, 15 kV and 4 mM β-CD. Furthermore, the relationships between migration times (t_m) of the analytes with separation temperature (T_s) or voltage (V) were also investigated. The equations and regression results describing the dependence of migration time on separation temperature and voltage are

Table 1
Regression data (n = 3) of ln (t_m) versus T_s and ln (t_m) versus ln (1/V)[a]

Compound	ln (t_m) versus T_s		ln (t_m) versus ln (1/V)	
	Equation	R	Equation	R
GSH	ln (t_m) = 2.096−0.0201 T_s	0.9971	ln (t_m) = 4.993 + 1.137 ln (1/V)	0.9997
NBD-Cl	ln (t_m) = 2.153−0.0194 T_s	0.9965	ln (t_m) = 5.111 + 1.151 ln (1/V)	0.9996
GSSG	ln (t_m) = 2.121−0.0170 T_s	0.9963	ln (t_m) = 5.080 + 1.133 ln (1/V)	0.9997

[a] The units of t_m, T_s and V are min, °C and kV, respectively.

listed in Table 1, which indicated that ln (t_m) was linear dependent on ln (T_s) and ln (1/V).

3.2. Linearity, sensitivity and reproducibility of the method

For evaluation of the quantitative applicability of the method, six standard solutions of GSH and GSSG in the concentration range of 0.22–45.00 μM were analyzed under the optimum experimental conditions. The linear regression equations between the peak height (Y, RFU) and the concentration (X, μM) were obtained (Table 2). However, when the sample of tobacco leaves was considered, the peaks of GSH and GSSG were interfered by co-existing components, so the content of GSH and GSSG in tobacco leaf sample were determined by second-derivative electrophoregram [22]; the linear relationships between the concentrations and corresponding peak heights obtained in second-derivative electrophoregram are listed in Table 2. The detection limits (signal-to-noise ratio = 3) of GSH and GSSG obtained in normal and second-derivative electrophoregrams were also given. For GSH and GSSG under optimum conditions, the relative standard deviation (R.S.D., n = 5) of the migration time in normal and second-derivative electrophoregrams were consistent (0.55% for GSH and 0.7% for GSSG), because the migration times of the analytes in both kinds of electrophoregrams are same. The R.S.D. values of peak heights were 2.64 and 0.64% in normal electrophoregrams and 2.95 and 2.58% in second-derivative electrophoregrams, respectively. The results indicated that the method could be applied to the sensitive analysis of GSH and GSSG with good reproducibility. Fig. 1 shows the normal and corresponding second-derivative electrophoregrams of the standards.

3.3. Application

3.3.1. Analysis of human plasma sample

The method was applied to the analysis of GSH and GSSG in human plasma sample. The content of GSSG in the plasma sample was very low, but the content of GSH was very high, so the contents of GSSG and GSH were determined by normal electrophoregrams with derivative solutions before and after 10-fold dilution, respectively. The electrophoregram of plasma sample without dilution is illustrated in Fig. 2(a).

3.3.2. Analysis of tobacco leaf sample

For tobacco leaf sample, because GSH and GSSG were interfered by co-existing components, their contents and recoveries were determined by second-derivative electrophoregrams. Fig. 2(b) shows the second-derivative electrophoregrams of tobacco leaf sample after two-fold dilution.

The peaks of GSH and GSSG were identified with adding standards to the samples. The determination results and the recoveries are all listed in Table 3.

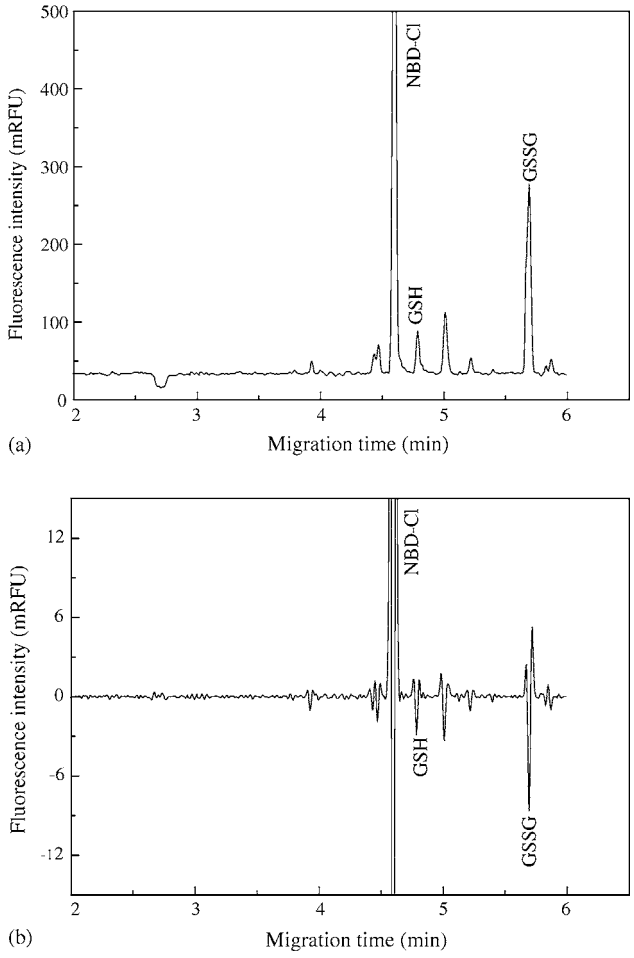

Fig. 1. Normal (a) and second-derivative (b) electrophoregrams of the standards. Derivatization conditions: 20 mM borate (pH 8.75), reaction at 50 °C for 20 min; separation conditions: 20 mM borate at pH 9.25, 20 kV, 25 °C, 4 mM β-CD, capillary length 47 cm (effective length 40 cm), sample injected for 5 s with a pressure of 0.5 psi.

Table 2
Regression data between peak height (Y, RFU) and corresponding concentration (X, μM) and the detection limits in normal and second-derivative electrophoregrams (n = 3)

Compound	Normal electrophoregram		Second-derivative electrophoregram	
	Equation	Detection limit (μM)	Equation	Detection limit (μM)
GSH	$Y = 0.0073\ X + 0.0103$ ($R = 0.9981$)	0.046	$Y = 0.00051\ X + 0.00075$ ($R = 0.9975$)	0.046
GSSG	$Y = 0.0553\ X + 0.0173$ ($R = 0.9989$)	0.012	$Y = 0.0031\ X + 0.00055$ ($R = 0.9996$)	0.014

Table 3
Determination results of GSH and GSSG in samples (n = 3)

Compound	GSH		GSSG	
	Content	Recovery (%)	Content	Recovery (%)
Human plasma sample	6.5 μmol ml^{-1}	98.4	8.1 nmol ml^{-1}	103.5
Tobacco leaf sample	0.58 μmol g^{-1}	106.7	0.070 μmol g^{-1}	96.2

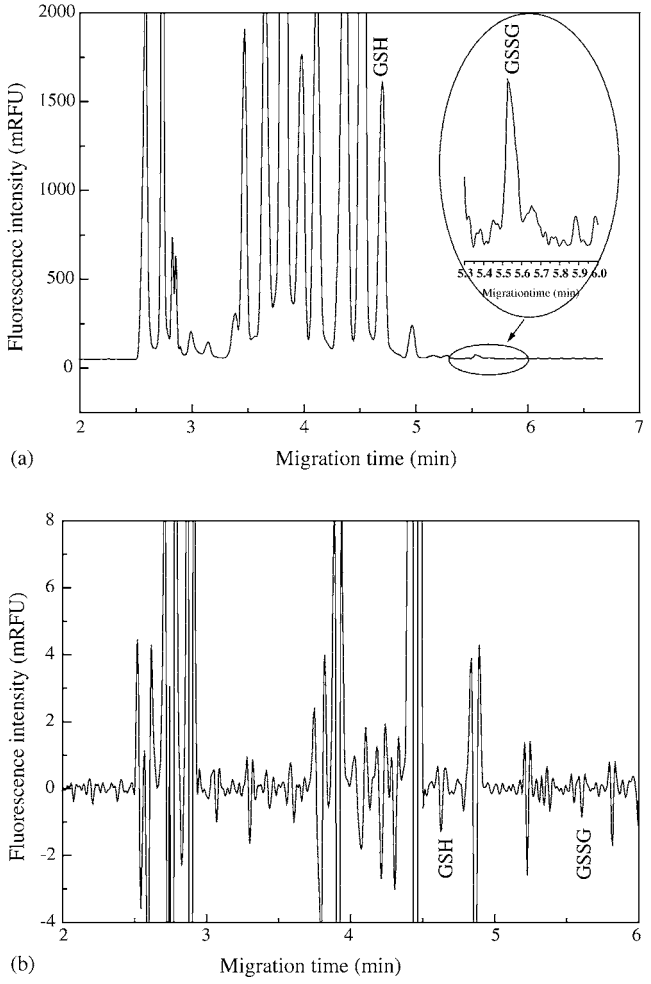

Fig. 2. Electrophoregrams of the samples (a) human plasma and (b) Tobacco leaf. Derivatization and separation conditions, see Fig. 1.

4. Conclusion

A novel CE-LIF method is developed for the analysis of GSH and GSSG. The results show that the method can be applied to the analysis of GSH and GSSG in human plasma and tobacco leaves with high sensitivity and good reproducibility. In addition, the results indicate that the analytes are sometimes partially interfered by co-existing components, but second-derivative electrophoregrams can resolve this problem without sacrificing the sensitivity.

Acknowledgements

The financial support of National Nature Science Foundation of Chinese (No. 20275014) is gratefully acknowledged.

References

[1] A. Meister, S.S. Tate, Annu. Rev. Biochem. 45 (1976) 559.
[2] A. Meister, M. Anderson, Annu. Rev. Biochem. 52 (1983) 711.
[3] P. Reichard, L. Thelander, Annu. Rev. Biochem. 48 (1979) 133.
[4] R.J. Bridges, J.Y. Koh, C.G. Hatalski, C.W. Cotinan, Eur. J. Pharmacol. 192 (1991) 199.
[5] F.J.T. Staal, M. Roederer, L.A. Herzènberg, L.A. Herzenberg, Proc. Natl. Acad. Sci. USA 87 (1990) 9943.
[6] T. Kalebic, A. Kinter, G. Poli, M.E. Anderson, A. Meister, A.S. Fauci, Proc. Natl. Acad. Sci. USA 88 (1991) 986.
[7] M. Roederer, S.W. Ela, F.J.T. Staal, L.A. Herzènberg, L.A. Herzenberg, AIDS Res. Hum. Retroviruses 8 (1992) 209.
[8] A. Meister, Cancer Res. 54 (1994) 1969s.
[9] J.L. Kevin, T. Hélène, J.R. Wanger, Anal. Biochem. 274 (1999) 125.
[10] C. Muscari, M. Pappayallo, D. Ferrari, E. Giordano, C. Capanni, C.M. Caldarera, C. Guarnieri, J. Chromatogr. B 707 (1998) 301.
[11] E. Camera, M. Picardo, J. Chromatogr. B 781 (2002) 181.
[12] T. He, D. Quinn, E. Fu, Y.K. Wang, J. Chromatogr. B 727 (1999) 43.
[13] O. Orwar, H.A. Fishman, N.E. Ziv, R.H. Scheller, R.N. Zare, Anal. Chem. 67 (1995) 4261.
[14] C. Chassainy, J.G. Gonin, C.S. Wilcox, I.W. Wainer, J. Chromatogr. B 735 (1999) 219.
[15] S.H. Kang, W. Wei, E.S. Yeung, J. Chromatogr. B 744 (2000) 149.
[16] T. Toyo'oka, J. Tanabe, Y. Kashihara, Anal. Chim. Acta 433 (2001) 1.
[17] E. Caussé, N. Siri, H. Bellett, S. Champagne, C. Bayle, P. Valdiguié, R. Salvayre, F. Couderc, Clin. Chem. 45 (1999) 412.

[18] P.B. Ghosh, M.W. Whitehouse, Biochem. J. 108 (1968) 155.
[19] L.M. Preston, M.L. Weber, M. Murray, J. Chromatogr. B 695 (1997) 175.
[20] E.T. Wise, N. Singh, B.L. Hogan, J. Chromatogr. A 746 (1996) 109.
[21] J.Y. Zhang, J.P. Xie, X.G. Chen, Z.D. Hu, Analyst 128 (2003) 369.
[22] S.H. Liu, X. Tian, X.G. Chen, Z.D. Hu, J. Chromatogr. A 928 (2001) 109.