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Sensitive determination of glutathione in biological samples by capillary electrophoresis with green (515 nm) laser-induced fluorescence detection☆

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ABSTRACT

A new sensitive capillary electrophoretic method with laser-induced fluorescence (LIF) was developed for quantitation of glutathione (GSH) in biological samples. Eosin-5-maleimide was used to label the GSH molecule and the formed conjugate was separated in a 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid electrolyte at pH 7.0 in less than 3 min. The conjugate was detected with an in-house built LIF system, utilizing an inexpensive 515 nm diode laser module. Studies were performed to optimize the derivatization (the ratio of reagent to analyte, the reaction time, pH, etc.) and separation conditions. Sensitive detection of GSH at concentrations as low as 0.18 nM was obtained. The method was applied in the analysis of biological fluids (exhaled breath condensate, saliva) and was found to be suitable for determination of GSH in these samples at trace levels below 1 nM. To the best of our knowledge, this is the first report on determination of GSH in exhaled breath condensate by capillary electrophoresis (CE).

1. Introduction

Reduced glutathione (GSH) is a major tripeptide occurring in animal and human tissues and fluids [1]. The biologically active site of GSH is represented by the reactive thiol group of the cysteine residue, which is highly nucleophilic and facilitates the role of GSH as a free radical scavenger both under normal physiological conditions and in deactivation and elimination of xenobiotics [2,3]. By reacting with radicals and oxygen-reactive species, it fulfills a major role in protecting the cells and tissue structures [4]. GSH is believed to be involved in various diseases, such as atherosclerosis, rheumatoid arthritis, or adult respiratory distress syndrome, in which its normal concentration usually decreases and on the other hand the concentration of the oxidized form (GSSG) increases [5].

The concentration of GSH in biological fluids covers several orders of magnitude, typically from nM to mM. For instance, GSH concentration in whole blood is in the mM range [6] and is about 2–3 orders higher than in blood plasma or serum. In saliva, the concentration of GSH is about the same as in the serum samples but only nanomolar concentration of GSH was detected in exhaled breath condensate [7].

Sensitive analytical methods are, therefore, needed to determine low concentrations of GSH in many biological fluids, also due to the need for dilution during sample preparation. Conventional spectrophotometric method developed by Tietze in 1969 [8] is still one of the most widely applied techniques to detect GSH due to its simplicity, satisfactory sensitivity, and low cost. Spectrofluorimetric methods have been developed to improve the sensitivity of GSH and GSSG determination in different matrices [9–12]. Nevertheless, due to their low specificity, the spectrophotometric or spectrofluorimetric methods cannot compete with separation methods such as HPLC or CE. The separation methods extend the applicability to multiple thiol compounds that have physiological importance (for instance, cysteine, homocysteine, etc.). A number of HPLC and ultra-performance liquid chromatography (UPLC) methods have been developed for the determination of GSH, GSSG, and related compounds [6,13,14]. Detection systems include UV absorbance, fluorescence, electrochemical, mass spectrometry (MS), tandem mass spectrometry (MS/MS), and atomic fluorescence spectrometry (AFS). UV absorbance or conductometric detection methods usually do not require derivatization, but the price paid for the

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simplicity is their poor sensitivity. Therefore, mainly fluorescence detection has been used in the analysis of GSH by HPLC and other separation techniques, such as CE.

Sample preparation is often mentioned as one of the most critical steps in the GSH determination because GSH is easily oxidized during the sample preparation [15]. For instance in the analysis of blood samples, the most important source of artifacts associated with sample preparation that may influence the results of GSH and GSSG assays is the hemolysis of the red blood cells [16] and improper sample storage [17]. Hemolysis can cause either overestimation of GSH in the plasma because the GSH level in erythrocytes is about 500-fold higher than that in plasma, or, without refrigeration, lead to the underestimation of GSH and overestimation of GSSG when autoxidation and proteolysis of GSH in the plasma are not repressed [18–20]. The protein removal prior to the analysis by acidification or by addition of an organic solvent, such as ACN, acetone, or methanol, can also cause autoxidation of GSH, unless the thiol group is protected. Rossi et al. revealed that the restoration of neutral–alkaline pH in acidified samples also leads to a rapid decrease of thiol concentration if no previous treatment with thiol-masking agents has been performed [19,21]. The use of filters excluding macromolecules on the basis of their pore dimensions is an alternative method to remove proteins [22,23], but large volume of sample is required. Therefore, ideally, an analytical method should be able to detect very low concentrations of GSH in biological samples with no or minimum sample pretreatment.

The need for extensive sample pretreatment still remains a bottleneck of most HPLC methods, because various matrix compounds can interact irreversibly with the stationary phase. Not so much for capillary electrophoresis (CE) that is less prone to matrix effects, has high resolution and is amenable for low sample volumes (advantageous for most biological samples). The low sensitivity of UV [24–26] and conductivity detection in GSH analysis [27], however, applies also for CE. Use of electrochemical detection methods [28,29] has also been reported for analysis of GSH and related compounds, but laser-induced fluorescence (LIF) detection is much more widespread in CE, because very low LODs can be achieved [30]. Numerous CE methods have been reported for determination of GSH in biological samples after derivatization with monobromobimane [31], 5-iodoacetamidofluorescein [32–37], 6-iodoacetamidofluorescein [38–40], 2,3-naphthalenedicarboxaldehyde [41–45], 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole [46], 5-bromomethylfluorescein [47], fluorescein-5-maleimide [48], 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole [49,50], and o-phthaldialdehyde [51].

Majority of the CE–LIF methods have been developed for Ar-ion lasers and excitation wavelength of 488 nm with commercially available CE–LIF instruments (Beckmann Coulter Inc., CA, USA) or CE detectors (Picometrics Technologies SAS, Toulouse, France).

CE–LIF can benefit from latest technological advances, as many off-the-shelf laser modules with various wavelengths are available. Semiconductor lasers emitting at wavelengths in the visible range (for instance 515, 520, 532, 635 nm, etc.) are becoming widely available, they are inexpensive, durable, small in size, and operate at low voltages. More flexible choice of excitation wavelength allows better matching to absorption maxima of existing and newly synthesized fluorescent tags. Higher laser wavelengths are beneficial in analysis of biological samples due to the potential lower fluorescence interference of the unwanted matrix compounds.

In this article, we describe the use of 515 nm green laser module and CE–LIF for sensitive determination of GSH in various biological samples: exhaled breath condensate (EBC) and saliva. Eosin-5-maleimide (EMA) is used as a fluorescent tag for GSH derivatization. This novel reagent that has not been previously used in CE–LIF studies of GSH has a maximum of the excitation spectra exactly at 515 nm matching the employed laser module, and thus, yielding limits of detection in the subnanomolar range. We demonstrate, for the first time, that GSH can be quantified in as little as 20 μl of EBC sample.

2. Experimental

2.1. Apparatus

Electrophoretic system. A laboratory-built CE instrument was employed for all electrophoretic separations. The separation voltage of +15 kV was provided by a high-voltage power supply unit (Spellman CZE2000R Start Spellman, Pulborough, UK). Two Pt wires (0.5 mm id, 3 cm length, Advent Research Materials Ltd., Eynsham, England) were used as electrodes. Fused-silica (FS) capillaries (50 μm id, 375 μm od, 50 cm total length, Polymicro Technologies, Phoenix, AZ, USA) were used for the separation. Prior to the first use, the separation capillary was preconditioned by flushing with 1 M NaOH for 10 min, deionized double distilled (DI-DD) water for 10 min and BGE solution for 10 min. Between two successive injections, the capillary was flushed with BGE solution for 1 min. At the end of a working day, the capillaries were flushed with DI-DD water for 15 min, followed by applying vacuum for 5 min and stored dry overnight. All CE experiments were performed at ambient temperature.

Injection. Injection of standard solutions and EBC samples was carried out hydrodynamically. The injection capillary end was immersed in a sample vial and elevated to a height of 10 cm for 35 s.

Detection system. A home-built LIF detector was assembled and optimized for this work as described previously with slight modification [52]. In brief, a direct emitting diode laser module operated in CW mode with nominal wavelength 515 nm and power ~4 mW, (CW 520-05, Roithner Laser Technik, Vienna, Austria) was used. The laser power was measured with NOVA II (Ophir Optronics Ltd., Jerusalem, Israel). The laser beam was focused onto the separation capillary using a double convex lens (focal length: 1 cm, Edmund Scientific, NJ, USA). Fluorescence was taken orthogonally by a 60× microscope objective with working distance 0.65 mm (Edmund Scientific, NJ, USA) and focused on a photomultiplier tube (model R6356, Hamamatsu Photonics, Germany) through an optical filter (540 nm long pass filter (3RD540LP Omega Optical, VT, USA)) and a 0.4 mm optical slit. The photomultiplier tube (PMT) was operated at a voltage of 560 V using a power supply (model C4900, Hamamatsu Photonics, Germany). Current from the PMT was converted into voltage using an RC circuit (R = 1 MΩ, C = 10 nF) and digitized with A/D 16-bit converter PC card (PCA-1608A, Tedia, Czech Republic). An interface programmed in LabVIEW v6.0 (National Instruments, TX, USA) was used to control the instrument and to collect and display CE data.

2.2. Chemicals

All chemicals were of reagent grade and DI-DD water (obtained from Masaryk University facilities, Brno, Czech Republic) was used for stock solution preparation. Reduced glutathione (GSH, ≥98% purity) was purchased from Sigma-Aldrich (Steinheim, Germany). GSH stock solution was prepared fresh each day in DI-DD water and diluted to the required concentration. The separation electrolyte (BGE) for CE measurements was prepared weekly as a 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) solution (99% purity Merck, Germany) in DI-DD water. pH was adjusted by 1 M NaOH (JT Baker, USA). EMA (purity ≥ 85% at 254 nm) was purchased from Molecular probes (Eugene, OR, USA). A 1 mM stock solution was prepared by dissolving the EMA in *N*,*N*-dimethylformamide (DMF, Sigma, Steinheim, Germany,