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Simultaneous quantification of reduced and oxidized glutathione in plasma using a two-dimensional chromatographic system with parallel porous graphitized carbon columns coupled with fluorescence and coulometric electrochemical detection

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Abstract

A method for the simultaneous quantification of reduced and oxidized glutathione in human plasma employing a two-dimensional chromatographic system with parallel porous graphitized carbon (PGC) columns coupled with fluorescence (FLD) and coulometric electrochemical detection (ED) has been developed. Post-sampling oxidation of reduced glutathione (GSH) was prevented by derivatizing the –SH group with monobromobimane (MBB) and the glutathione-bimane adduct (GSMB) was detected by FLD. Oxidized glutathione (GSSG) was detected by ED optimized to give lowest possible limits of detection (LOD). The method is fully validated and is currently used for determination of GSH, GSSG and its redox potential in different clinical studies.

Keywords: Glutathione; Electrochemical detection; Hypercarb; Plasma; GSSG; Chromatography

1. Introduction

Glutathione (γ-glutamyl-cysteinyl-glycine; GSH) is the major nonprotein thiol involved in the antioxidant cellular defence (Fig. 1) [1,2]. This tripeptide is synthesized in two sequential steps from cysteine, glutamate, and glycine by two cytosolic enzymes namely, γ-glutamylcysteine synthetase and glutathione synthetase [3]. GSH is among the most important antioxidant in cells involved enzymatically in reduction of hydroperoxides and nonenzymatically to maintain Vitamin E and Vitamin C in reduced and functional forms [1,4]. In doing so GSH is oxidized to GSSG (Fig. 1) which is either reduced enzymatically by glutathione reductase or excreted from cells into extracellular fluids. GSH also reacts with xenobiotics through the action of glutathione *S*-transferase (GST) and thus is involved in removal of xenobiotics from the cells. Since the concentration of GSH is much higher than cysteine in both cells and tissues, it serves as a reservoir for cysteine that can be toxic in higher concentrations. In addition, GSH is a major redox buffer in cells providing reducing environment for various proteins [5–7]. Several pathological conditions are characterized by GSH deficiency or an imbalance in the GSH/GSSG ratio [8–19].

Buettner and Schafer [20] has emphasized the importance of the redox environment of the cell viewed through the redox state of the GSH/GSSG couple. In order to measure the redox state of the GSH/GSSG couple one needs to determine the concentration of both GSH and GSSG in the particular biological sample. In cells, the concentration of GSH is around 1–10 mmol/L, whereas in plasma the concentration is 1–5 μmol/L. During oxidative stress in tissues the concentration of GSH decreases and the concentration of GSSG increases which is further associated with a decreased export of GSH and an increased export of GSSG to plasma. Thus, the oxidative stress in tissues can be expected to alter the redox state of the plasma pool. Because

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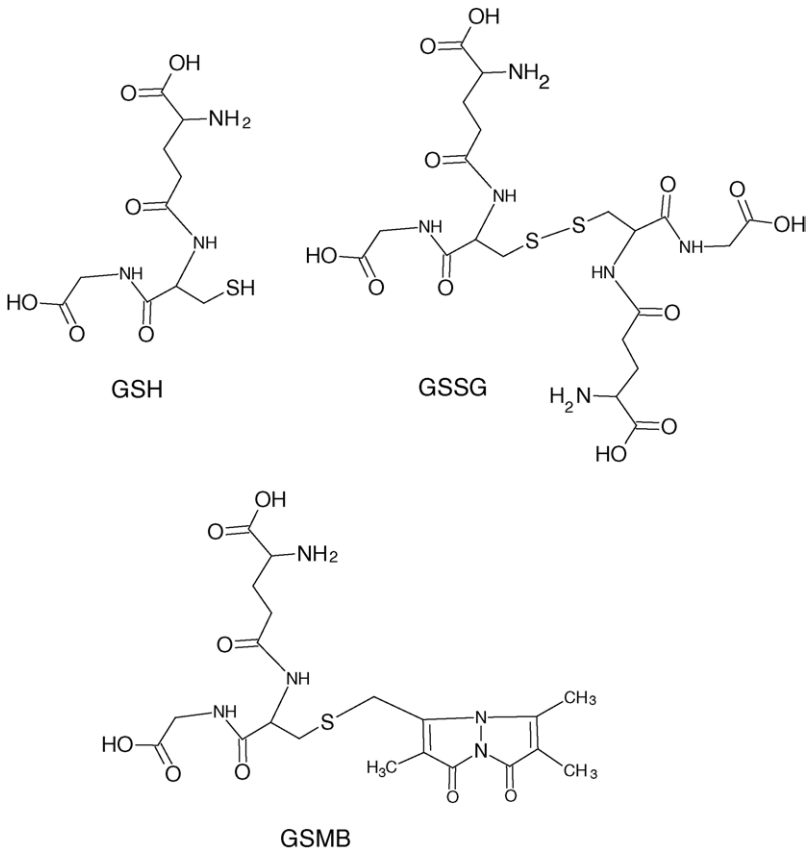

Fig. 1. Structures of reduced glutathione (GSH), oxidized glutathione (GSSG) and GSH bound to MBB (GSMB).

plasma glutathione concentration is likely to reflect GSH status in other less accessible tissues, measurement of both GSH and GSSG in plasma has been considered essential as an index of whole body glutathione status and a useful indicator of disease risk in humans [21].

Methods to measure GSH and GSSG in various biological samples have been reviewed recently [22,23]. The early techniques for the measurement of GSH and GSSG were based on an enzymatic recycling method by Tietze et al. [24]. The technique is simple but two separate measurements are required for determination of both GSH and GSSG. It also suffers from interference from high concentrations of reduced or oxidized cysteine [25], xenobiotics, unknown endogenous substrates [25–27] as well as *N*-ethylmaleimide (NEM) [28]. To overcome this drawback, one has to add known amount of GSSG to the sample (internal standard), which means that for GSSG every sample requires two separate estimations [29]. As a result, the technique is laborious and time consuming when a large number of samples are to be analyzed. GSH and GSSG concentrations measured by recycling method in plasma in healthy subjects also show large variations. GSH varies from 2.79 to 11.36 μmol/L and GSSG varies from 0.2 to 5.4 μmol/L [19,29–33].

Attempts to measure both GSH and GSSG simultaneously by chromatographic techniques in plasma are few [34–37]. Apart from Kong et al. [37], these studies used plasma samples from healthy individuals when measuring GSH and GSSG concentrations. Large variations in normal values were reported since GSH concentrations ranged from <8 nmol/L to 2.09 μmol/L and GSSG concentrations ranged from <10 nmol/L to 1.5 μmol/L.

Accurate and precise measurement of plasma GSH and GSSG is not straight forward because of the following reasons, (i) GSH can be easily oxidized during sample preparation giving erroneous results, (ii) plasma values are easily increased by incidental and non reproducible leakage from RBC (hemolysis) having 1000-times higher GSH concentration than plasma [38], (iii) the concentration of GSSG in plasma is most likely very low (nM range) and GSSG does not possess specific detection properties, (iv) γ-glutamyl transpeptidase (GGT) can degrade GSH if the enzyme is not inhibited during sample preparation [39].

Most of the previous methods focus only on stopping the oxidation of GSH either by acidifying the sample or by blocking the –SH group of GSH with an appropriate reagent [22,23]. But it is very important to have a remedy or solution for all the points mentioned above to accurately determine in vivo concentration of GSH and GSSG in plasma with high precision. For example, it has been shown that total glutathione (tGSH) was 53% lower in plasma when GGT was not inhibited [39].

Jones et al. [34,40] have measured GSH, GSSG, cysteine and other mixed thiols in a single run and have taken care of all the significant parameters that will minimize oxidation and degradation of GSH. However, the described method have the disadvantage of long derivatization times especially for GSSG (16–26 h) at room temperature and makes use of iodoacetic acid