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Determination of reduced and oxidized glutathione in biological samples using liquid chromatography with fluorimetric detection

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Abstract

A HPLC method for determination of both reduced (GSH) and oxidized (GSSG) glutathione in plasma, whole blood and rat hepatocytes has been developed and evaluated. Reduced glutathione reacts with orthophthaldehyde (OPA) to form a stable, highly fluorescent tricyclic derivate at pH 8, while GSSG reacts with OPA at pH 12. At measurement of GSSG, GSH was complexed to *N*-ethylmaleimide. For the separation, reverse phase column Discovery C_{18}, 150 mm × 4 mm, 5 μm, was used. The mixture of methanol and 25 mM sodium hydrogenphosphate (15:85, v/v), pH 6.0, was used as mobile phase. The analytical performance of this method is satisfactory for both GSH and GSSG. The intra-assay coefficients of variation were 1.8 and 2.1% for whole blood, 2.0 and 1.9% for rat hepatocytes, 4.3 and 5.2% for plasma. The inter-assay coefficients of variation were 5.8 and 6.2% for whole blood, 6.6 and 7.1% for rat hepatocytes, 6.9 and 7.8% for plasma. The recoveries were as follows: 98.2% (CV 3.5%) and 101.5% (CV 4.2%) for whole blood, 99.1% (2.5%) and 102.3 (4.4%) for rat hepatocytes, 94.1% (CV 7.5%) and 103.5 (CV 8.5%) for plasma. The calibration curve was linear in the whole range tested. The limit of detection was 14.0 and 5.6 fmol, respectively. The preliminary reference ranges of reduced and oxidized glutathione in a group of blood donors are (4.69 ± 0.93) and (0.28 ± 0.12) μmol/g Hb for whole blood, (1.82 ± 0.55) and (0.154 ± 0.044) μM for plasma.
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1. Introduction

The tripeptide, glutathione (γ-glutamyl-cysteinyl-glycine) can be obtained from the diet or can be synthesized de novo in the liver [1–4]. It plays important roles in biological systems. Glutathione (GSH) can readily be oxidized to its disulfide (GSSG) and the ratio of both forms is crucial for the characterization of the oxidative stress in cells. It also plays a role in the regeneration of other antioxidants. It can regenerate ascorbic acid from dehydroascorbic acid and membrane bound α-tocopherol from the α-tocopheryl radical formed during inhibition of lipid peroxidation [5,6]. Glutathione is important in the detoxification of potentially harmful endogenous compounds and xenobiotics (e.g. α-oxoaldehydes, monoamines, polyphenoles and some drugs). It acts as cofactor for enzymes including glutathione peroxidase, and other peroxidases, dehydrochlorinase, formaldehyde dehydrogenase, glyoxylase, maleyl-acetonase isomerase, and prostaglandin endoperoxidase isomerase [7,8]. The multifunctional properties of glutathione are reflected by the growing interest in this tripeptide.

Several methods are available for the determination glutathione in biological samples. Glutathione is measured after protein precipitation by spectrophotometry, fluorometry or by HPLC. Oxidation of GSH during sample preparation represents a major problem. The blocking of thiol group with various agents such as *N*-ethylmaleimide (NEM) iodoacetic acid and 2-vinyl pyridine is often used to prevent this phenomenon [9].

The most widely used technique, enzyme recycling, measures total glutathione (GSH and GSSG) in a reaction involving NADPH, 5,5′-dithiobis-(2-nitrobenzoic acid) and glutathione reductase [10].

A variety of HPLC techniques have also been developed. HPLC with ultraviolet detection requires derivatization [11–16]. With regard to poor limit of detection it may not

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be sensitive enough for some biological samples. HPLC with fluorescence detection requires derivatization with 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F) [17–20], ortophthalaldehyde (OPA) [21–25], monobromobimane [26–30], dansyl chloride [31] or 5-methyl-(2-(*m*-iodoacetylaminophenyl) benzoxazole) (MIPBO) [32]. HPLC with electrochemical detection, using of either amperometric or coulometric electrodes, can measure GSH and GSSG directly. These techniques avoid typical problems associated with derivatization procedures. Coulometric detection offers superior sensitivity and selectivity to the dual-amperometric approach. However, the measurement of GSSG requires a relatively high applied oxidation potential, shortening the life of the operating electrochemical cell [33–46]. Quite recently liquid chromatography/mass spectrometry assays were developed for the determination of glutathione [47–49] or glutathione conjugates [50].

The aim of this study was to develop rapid, simple HPLC method for measurement both GSH and GSSG in different biological samples, suitable for clinical trials.

2. Experimental

2.1. Reagents and chemicals

Reduced glutathione, oxidized glutathione, *N*-ethylmaleimide, orthophthalaldehyde (OPA), sodium hydroxide, hydrochloric acid, sodium hydrogenphosphate, metaphosphoric acid, EDTA, type I collagen and trypan blue were obtained from Sigma–Aldrich, medium William's E without phenol red, fetal bovine serum, penicillin, streptomycin and glutamine from Pan Biotech GmbH, HPLC-gradient grade methanol from Merck KgaA. Collagenase crude was obtained from SEVAC (Prague, Czech Republic) and insulin (Actrapid, Novo Nordisk), glucagon (Glukagen, Novo Nordisk), prednisolone (Solu–Decortin, Merck) were from supplier mentioned in brackets. All the others chemicals were of analytical grade.

GSH and GSSG solutions were prepared daily in 1 mM hydrochloric acid and stored at 4 °C until used.

2.2. Instrumentation

Chromatographic analysis was performed with a liquid chromatograph (Shimadzu, Kyoto, Japan), LC-10ADvp solvent delivery system, SIL-10ADvp autosampler, CTO-10ASvp column oven, RF-10Axl fluorescence detector and SCL-10Avp system controller. Data were collected digitally with CSW 32 chromatography software (DataApex, Prague, Czech Republic).

2.3. Animals

Male albino Wistar rats (BioTech, Konárovice, Czech Republic) were housed at 23 ± 1 °C, $55 \pm 10\%$ relative humidity, air exchange 12–14 times/h, and 12 h light/12 h dark cycle periods (6:00 a.m. to 6:00 p.m.). The animals had free access to standard laboratory rat chow (DOS 2B, Velaz) and tap water. All animals received care according to the guidelines set by the Institutional Animal Use and Care Committee of the Charles University, Prague, Czech Republic.

2.4. Hepatocyte culture

Hepatocytes were isolated from rats mentioned above with the body mass of 230–270 g by collagenase perfusion [51,52]. The viability of freshly isolated hepatocytes was more than 90% as confirmed by trypan blue exclusion. Isolated hepatocytes were suspended in William's E medium supplemented with fetal bovine serum (10%), glutamine (2 mM), penicillin (100 IU/ml), streptomycin (10 mg/ml), insulin (0.08 IU/ml), prednisolone (0.5 μg/ml), glucagon (0.008 μg/ml) and plated in collagen-coated Petri dishes (60 mm) at a density of 2×10^6 cells/Petri dish. Hepatocytes were allowed to attach in a gassed atmosphere (5% CO_2) at 37 °C for 2 h. At the end of the incubation period, the medium and hepatocytes were collected for determination of both reduced and oxidized glutathione.

2.5. Subject and samples

Samples of peripheral venous blood with EDTA as anticoagulant were obtained from a group of healthy blood donors ($n = 70$, 35 women and 35 men in the age 27–61 years, mean age 41 years). None of the participants had a serious or chronic disease and took any medications on the day of blood sampling. A written informed consent was obtained from all participants before starting the protocol and the study was approved by the Hospital Committee on Human Research (Regional Hospital of Pardubice, Czech Republic). Plasma was separated from red blood cells by centrifugation ($1700 \times g$, 15 min, 8 °C).

2.6. Sample preparation

Cold 10% metaphosphoric acid was carefully added (400 μl) to plasma, whole blood, hepatocyte samples or standards (200 μl). After incubation (4 °C, 10 min) and centrifugation ($22{,}000 \times g$, 15 min, 4 °C) supernatants were transferred into 1.5 ml propylene tubes (50 μl for determination of GSH and 200 μl for determination of GSSG) and immediately stored at −80 °C.

2.7. Derivatization procedure

Derivatization procedure was performed by a slight modification of the method of Hissin and Hilf [53].

2.7.1. GSH assay

To 50 μl of the supernatant, 1.0 ml of 0.1% EDTA in 0.1 M sodium hydrogenphosphate, pH 8.0, was added. To 20 μl portion of this mixture, 300 μl of 0.1% EDTA in 0.1 M sodium hydrogenphosphate, and 20 μl of 0.1% OPA in methanol, was added. Well-capped tubes were incubated at 25 °C for 15 min in dark. The reaction mixture was then filtered through a 0.20 μm nylon filter (4 mm diameter, Supelco, USA) and stored at 4 °C.

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