Glutathione: methods of sample preparation for chromatography and capillary electrophoresis

Ewelina BŁOŃSKA-SIKORA, Jerzy OSZCZUDŁOWSKI, Zygfryd WITKIEWICZ, Dariusz WIDEŁ - Institute of Chemistry, Jan Kochanowski University in Kielce

Please cite as: CHEMIK 2012, **66,** 9, 929-942

Abbreviations: GSH- reduced glutathone, GSSG- oxidized glutathone, SH- thiol group, K2EDTA- ethylenediaminetetraacetic acid dipotassium salt, K3EDTA- ethylenediaminetetraacetic acid tripotassium salt, NMR- nuclear magnetic resonance, SDS-PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis, MALDI-ToF- matrix-assisted laser desorption/ionization, TCA- trichloroacetic acid, NADHP-reduced form of nicotinamide adenine dinucleotide phosphate, NEM- N-ethylmaleimid, IAA- iodoacetic acid, DTT- dithiothreitol, ME- β-mercaptoethanol, MBB- monobromobimane, OPA- ortho-phtalaldehyde, BH- borohydride, TBP- tri-*n*-butylphosphine, TPP- triphenylphosphine, TCEP- tris (2-carboxyethyl) phosphine, DTNB- 5,5'-dithiobis-(2-nitrobenzoic acid), IA- iodoacetamid, VP- 2-vinylpyridine, DTPD- 4,4'-dithiopiridine, CMQT- 2-chloro-1-methylquinolinium tetrafluoroborate, SBD-F- 4- ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate, ABD-F- 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole, DBD-F-4-(*N*,*N*dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole.

Introduction

Glutathione (γ-glutamyl-cysteinylglycine) plays a lot of useful functions in human body and therefore determination of this small molecule is very important for present-day medicine and pharmacy. It is synthesized in every procariotic and eucariotic cell because it takes part in protection against oxidative stress, plays important role in detoxification and immunity modulation. Thanks to the glutathione the body is able to protect itself against different infections and cancer development, the liver has ability to detoxify heavy metals, toxins and other xenobiotics and cells are not subject of continuous destruction.

Characteristic element of glutathione structure is thiol group (-SH), which is responsible for biological functions of this compound. Because of the presence of this group, glutathione can occur in several forms. The most important forms are: reduced (GSH) and oxidized (GSSG) glutathione.

Other widespread forms are: S-nitrosoglutathione and conjugates of GSSG and proteins.

Reduced form of glutathione plays the principal role in human body as it protects organism against oxidative stress and negative impact of oxidants, for example reactive forms of oxygen (hydrogen peroxide, organic peroxides), egzogenous and endogenous electrophilic substances and also oxidized forms of other antioxidants for example vitamins E and C. Molecule of glutathione is also presented in very specific selenoenzym- glutathione peroxidase, which is the most important antioxidant protecting organism from hydrogen peroxide and lipid peroxides. GSH antioxidative activity appears also in reduction of thiol groups of different amino acids and protects them from irreversible oxidation to sulfonic or sulfinic acid and losing their activities.

Moreover, glutathione can be bound to the proteins, leading to the formation of glutathionylated proteins [1]. During the process of glutathionylation, the thiol groups of amino acids are bounded to the molecule of glutathione. This kind of connection is able to stabilize some proteins and protect them from oxidative stress. Glutathionylation regulates also activity of enzymes and process of transcription. Nucleophosmin involved in assembly of ribosomal proteins and cyclophilin involved in proteosomal degradadation of proteins and cytoskeletal proteins belongs to the class of glutathionylated proteins [2].

The antioxidative properties of GSH are especially important for erythrocytes. Red blood cells are highly exposed to free radicals mostly because of presence of iron ion, which has different oxidation number [3, 4].

The reduced glutathione/oxidized glutathione ratio (GSH/GSSG) is signed by R symbol. This is used to evaluate oxidative stress status in biological systems, and alterations of this ratio have been demonstrated in several diseases and during aging. The value of GSH/GSSG in the liver in physiological conditions is 300-400, whereas during starvation this ratio decreases to 2. This is very useful tool for determination of damage of different organs and tissues.

In human beings both GSH depletion and growth are linked to a number of diseases states but mostly acquired or congenital deficiency is observed. GSH depletions are associated with pathological conditions in organism such as: rheumatoid arthritis, muscular dystrophy, alcoholic liver damage disease, glaucoma, neurodegenerative diseases (Alzheimer disease, Parkinson disease, multiple sclerosis), schizophrenia, autism, arteriosclerosis, diabetes, asthma, chronic obstructive pulmonary [5÷8]. Moreover, glutathione concentration measurement represents an important tool for diagnosis of γ-glutamyl cycle disorders. Patients who exhibit deficiency of GSH have low concentration of specific enzymes: glutathione synthetase and γ-glutamylocysteine synthetase in erythrocytes and whole organism [2].

Sort of matrixes for glutathione determination

Glutathione concentration in human beings and animals has been analyzed mostly in plasma, whole blood and erythrocytes, because GSH to GSSG ratio in those structures represents sensitive indicator of oxidative stress in whole organism. Biological samples have been also collected from different tissues and cells such as: gastric mucosa [9] mitochondria from lymphocytes and granulocytes [10] human promyelocytic leukemia cell line HL-60 [11] lung tissue [12] kidneys, spleen and brain [13]. Glutathione has been also analyzed with good result in plants tissues for example in maize seedlings [14] and in Brassica juncea [15].

Sample preparation

The critical issue during analysis of amino thiols represents sample preparation, because they have huge oxidoreductive activity. Attention must be paid on blood withdrawal, refrigeration during sample processing and, for plasma determinations, immediate centrifugation of the samples. Long isolation time of erythrocytes from blood, preparation of different tissues and separation of subcellular organelles are accountable for errors in the determination of GSH and GSSG.

GSH undergo non-enzymatic autoxidation at pH >7, and enzymatic conversion of GSH. This step is mediated by γ-glutamyl transpeptidase which exhibit optimal activity at neutral pH. That compound has large oxidoreductive abilities because of huge reactivity of –SH group.

The measurement of GSSG concentration, especially in plasma also demands caution and special sample preparation. After withdrawal GSSG is degraded by proteolysis during less than 2 minutes [19]. Refrigeration during sample pretreatment and protein precipitation can minimize oxidation and proteolysis.

Of course, the differences in sample preparation stages depends on type of the tissue, form of glutathione and methodology of analysis, but there are some common bases for all methods. Sample preparation divides into several stages however each method does not have to contain all of them:

- sample collection and its protection
- precipitation of proteins
- blocking of free thiol groups, using alkaline or acidic reagents
- reduction of disulfides
- derivatization
- Sample collection.

The most important aspect during this procedure is to protect samples from coagulation process speeded up by calcium ions as a cofactor of the blood clotting cascade. This undesirable process is reduced by adding to fresh collected blood the small amount of chelating reagents such as K_2EDTA, K_3EDTA or heparin. Blood samples can be also collected to special tubes with chelator, protecting blood from coagulation induced by Ca^{2+} or thrombocytes during the contacts with plastic or glass surface of test tubes. That substances also entrap many transition metals (including Fe^{2+}) avoiding some oxidative reactions.

Glutathione is usually studied in plasma, which contains only 0.5% of the blood content, whereas erythrocytes contain 99.5%. Plasma concentration of GSH reflects concentration of this compound in whole organism [1]. Glutathione analyzing in human plasma brings some difficulties and improper sample preparing may completely alter analytical data. Because of to the fact that GSH concentration in red blood cells is significantly higher (500 times) than in plasma this is necessary to pay attention to protecting samples from haemolysis of erythrocytes. Even dissolution of 1% of red blood cells may drastically increase plasma concentration of GSH.

The second important aspect both during blood preparation and sampling other tissues and organs is protecting them from high temperature. A drastic decrease of GSH concentration has been observed during samples storage in room temperature as an effect of oxidation to GSSG even about several dozen percent after 5 minutes from blood collecting. The temperature of samples storage depends on time from preparation to chromatographic processing. Samples are frozen from -20°C to -80 °C.

Precipitation of proteins

The essential stage during the samples collection is elimination of some undesirable enzyme, for example γ- glutamyl transpeptidase, which is responsible for glutathione catabolism. To avoid complete decomposition of analyzed thiol compounds it is necessary to remove immediately this enzyme and also other proteins disturbing in analyze. The tissues with high concentration of γ-glutamyl transpeptidase such as: kidneys and spleen should be frozen immediately after collection and homogenization, whereas the tissues with low concentration of this enzyme such as liver, spleen and brain do not have to be homogenized. They should be frozen after collecting and storage at -20 °C. Direct injection of biological samples into the HPLC or CE system is not recommended [19]. For example, the presence of proteins in injected samples during CE analysis may led to adsorbing them on the capillary wall and affecting migration time, peak shape and detection response [20].

Only a few methods (e.g. NMR) do not demand deproteinization. Acidification, addition of an organic solvent, such as acetonitryl, acetone or methanol, and ultrafiltration are used for protein elimination [2]. Organic solvents are preferable during the analysis with mass spectrometer [19]. Ultrafiltration is a useful method to remove proteins because it does not require the addition of acids or organic solvents that can affect the separation, derivatization and detection. The separation of GSH and GSSG can also be achieved by applying membrane filtration in combination with centrifugal microconcentration [20].

Typically, to obtain plasma from blood samples it is necessary to acidify blood immediately after venipuncture [21]. Acidification causes precipitation of proteins including undesirable enzymes disturbing in analyze.

Acidification also gives possibility to measure total glutathione concentration in each tissue by releasing free glutathione from protein-GSH adducts [1]. There are two helpful techniques used in determination of glutathione conjugates with proteins (glutathionylated proteins) SDS-PAGE and MALDI-ToF analysis. However, they are qualitative or semiquantitative, what represents the main disadvantage of these procedures, thus the concentrations of the various protein–GSH adducts sometimes cannot be determined [2].

Precipitation has been handled with several acids such as trichloroacetic acid (TCA), trifluoroacetic acid, perchloric acid, sulfosalicylic acid, picric acid, metaphosphoric acid, yielding a clear, protein-free supernatant after centrifugation. Sulfosalicylic acid and picric acid are usually used at a concentration of 5% w/v, and metaphosphoric, chlorine (VII) and trichloroacetic acids at a concentration of 1% w/v. However the major problem in such determination is oxidation of thiols frequently leading to an overestimation of disulphides [13]. TCA seems to be the most useful deproteinization acid because it has been observed that only 3-4 % of GSH are oxidized within 20 h at 0°C after the addition of TCA to the sample [11]. 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.

Reduction of disulphides

The determination of free plus bound GSH and other low molecular-mass aminothiols, the reduction of the disulfide bonds formed between them and other thiols or proteins needs to be accomplished [19]. Precursor of GSH -cysteine can be detected only after reduction of all disulfides in biological sample Reduction can be achieved by: chemical reaction (adding reductants), electrolysis or enzymatic reactions (adding glutathione reductase or NADPH to the sample) [2, 22]. There are a variety of chemical reductants and the selection of the reducing reagent is very important for assay performance. For example, some reductants can react with labeling reagents what is a source of wrong results. Reduced glutathione can undergo reoxidation process before derivatization procedure what often leads to overestimation of GSSG and incorrect results.

This phenomenon can be avoided with thiol-masking agents, such NEM [11, 23-27] or IAA [19, 28]. Oxidation is also catalyzed by metal ions (especially iron and cooper), therefore using chelating agents like EDTA 1,10 fenantrolin [2] or deferoxamine [11] during sample collection is required. For some kind of analysis, it is necessary to remove completely the excess reducing agent before further modification, for example by gel filtration or acid precipitation. The reducing agents cannot also cross-react with derivative reagents during the next stage of analysis what often leads to wrong results of analysis. Reducing agent must be also compatible with the thiol-specific derivatization agent [29].

A quantitative elimination of reducing agent can be difficult to obtain with acid precipitation however the disadvantage of gel

filtration is regeneration of disulfides unless the buffer is kept at low pH. Furthermore, gel filtration could not be used during working with partially aggregated material [2, 30].

Thiol-containing reductants

This group contains such reductants as: dithioerythritol, dithiothreitol (DTT) and β-mercaptoethanol (ME).They are the most often used reductants in aminothiols analysis, highly specific. However the one of disadvantage of this group is that they can cross-react with detection agents such as monobromobimane (MBB) or *o*-phthalaldehyde (OPA), leading to the formation of interfering fluorescent compounds [2, 19].

The other inconvenience is fact, that at optimal for that reactions pH value above 7 oxidation can occur. The reagents are also sensitive to oxidation so they must be protected from oxygen and maintain with metal chelating agents.

ME belongs to weak reagents and must be used at very high concentration. ME is mainly used in the presence of sodium dodecyl sulfate [2]. This reagent was used as a redactor in many applications: UV, fluorometric, CE, LCMS. DTT is stronger reducing agent than ME, used mostly in spectrophotometric techniques. An important advantage of this reagent is its membrane permeability, which gives the possibilities of using it as a reductant in cellular systems.

N-acetyl-cysteine ethyl ester is strong disulfide bond-reducing thiol. This reagent was used for the quantitative analysis of GSH and GSSG in the cytosol of red blood cells as GSH-OPA derivative with fluorometric detection [31].

Sodium and potassium borohydride (BH) are a very strong, highly reactive reducing agents and that is why their solutions should be prepared immediately before use. They are also unstable in aqueous solution. Use of sodium borohydride may lead to problems with derivatization reaction because of pH control difficulties [28, 29]. Reduction of protein disulfides with high concentration of that reagents (1,4 mol/l) is complete after few minutes, while with the lower concentrations even 30 min.

BH is used in quantitative reduction of protein disulfides with or without the presence of denaturing agents and also in reduction of protein disulfides in whole cell extracts. The main advantage of BH is that excess reagent can be removed easily by the addition of acid or acetone. The solutions containing BH tend to foam but this problem can be solved by adding surface active compounds, for example octanol or hexanol [2,19].

Trialkylphosphines

This group has more advantages than reducing agents described above. Ttri-*n*-butylphosphine (TBP), triphenylphosphine (TPP) and tributylphosphine and tris (2-carboxyethyl) phosphine (TCEP) are strong reducing agents, active even in low concentration. Trialkylphosphines do not react with other functional groups of amino acids furthermore they are unreactive with thiol alkylating and derivatization agents. However they are quite reactive toward iodoacetic acid, iodoacetamide and NEM even at low pH, thus the reactions with phosphines must be carried out in a step separate from alkylation [2, 22].

TCEP is the most popular reagent in this group used in analysis. Its use may provide more reproducible assays than with TBP and TPP. This is water-soluble and nonvolatile reagent, used mostly for low-molecular disulfide bonds and surface-exposed protein disulfides reduction. The advantage of this reagent in comparison with thiol-containing reductants is its higher resistance to oxidation catalyzed by metal ions. However, TCEP can interfere with certain disulfide containing derivatizing agents, including 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) [19]. This reagent was used by many authors in different analysis [9, 29, 32]. Mono-, di- and trimethyl ester analogs are more reactive at lower pH and lipophilic than TCEP. They are also more reactive toward small-molecule [19]. The esterification increases membrane permeability, allowing the esters to penetrate phospholipid bilayers [2].

Derivatization

Every procedure of thiols analyzing except for vivo assays and those based on electrochemical and tandem mass spectrometry, require derivatization. Derivatization of GSH usually increases the method specificity and sensitivity. Derivatization reagent should react rapidly and specifically with thiol group at lowest possible temperature and weakly acidic pH to prevent oxidation of the analytes. Chromophore or fluorophore added to an analyte decrease the limits of detection or quantitation. Increase in sensitivity subsequent to derivatization result in improvement in chromatography delivers more analyte into the detector in a narrower chromatographic peak. Improvement in sensitivity is only one function of analytical derivatization, but also for the stabilization of thiols, ionization responses or induction of a charge [20]. Derivatives are usually more lipophilic than the analyte what is especially important for small molecules which are invariably hydrophilic [33].

Reagents for UV-VIS detection

Ultraviolet detection is commonly used technique in high performance liquid chromatography and capillary electrophoresis. Analytical methods using colorimetric reagents and UV absorbance detection are less sensitive, but simpler as compared with fluorescent or electrochemical detection. There are a number of thiol-reactive reagents, commercially available or made in laboratories which react with thiol functional group to produce UV absorbing derivative.

The compounds most commonly used are: maleimide-type reagents to which N-ethylmaleimide (NEM) belongs: iodoacetic acid (IAA) and iodoacetamid (IA), yielding thioethers [19]. NEM is also used in combination with glutathione reductase-coupled enzymatic recycling method [34-36], fluorescence detection and mass spectrometry for the measurement of GSSG as the thiol-masking agent. The application of NEM presents difficulties due to the inhibition of glutathione reductase by NEM. The reagent excess must therefore be eliminated either by solvent extraction or by solid-phase extraction [19].

Reactions with NEM are typically performed at concentration 1 M and below pH 7 or at pH values above 7 if incubation times are prolonged to more than 2 hours. The instability in alkaline media caused by hydrolysis of the maleimide ring and the fact that some low-molecular thiol adducts with NEM can undergo intramolecular transamidation to cyclic forms at pH values above 9 [2]. NEM also binds amino groups at pH >7.5, even if more slowly as compared with thiol functionalities and that is why during long incubations at alkaline pH, or if a derivatization agent of the amino group, such as 1-fluoro-2,4-dinitrobenzene is used the excess of NEM must be removed before the alkalization of the medium [20, 22]. Giustarini et all [37] developed the method based on the analysis of GSH conjugate with NEM. Blood samples treated with *N*EM were stable at −20 °C for 90 days.

IA and IAA are also used as the thiol-masking agents for the measurement of GSSG. These alklylating agents react irreversibly with aminothiols in a nucleophilic substitution reaction formed *S*-carboxymethyl and *S*-carboxamidomethyl derivatives. They are water-soluble and can be prepared at concentrations of 1 and 0.5 M, respectively at pH 8. They must be protected from light during storage. Unfortunately at neutral or alkaline pH they react with the hydroxyl group of tyrosine, the amino group of lysine and imidazole group of histidine. Besides, at low pH both reagents react with the sulfur atom of methionine. However, the SH group reacts much faster than any other group in the undesirable reactions. For example, IAA reacts with histidine 1000-fold slower than with cysteine. Thus, the alkylation procedure assuming the use of these reagents seems to be

thiol-specific. The disadvantages of IA in comparison with IAA are that the reagent is membrane impermeable and is inappropriate for thiols determination in vivo and it can form undesired products [2, 19].

Another alkylating agent is 2-vinylpyridine. This compound in contrast to NEM does not inhibit glutathione reductase and that is why is used as the alkylating agent in the DTNB–glutathione reductase recycling assay. The optimum value of pH for VP is between 5 and 8 and the reactions require high excess of this reagent and long incubation times [2]. Recently, 1-methyl-2-vinylpyridinium trifluoromethane sulfonate and 1-methyl-4-vinylpyridinium trifluoromethane sulfonate have attracted attention as thiol-masking agents but they were used only in spectrophotometric assays for analyzing thiols [19].

5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent) invented by Ellman in 1959 has been still the most common used reagent for the quantification of thiols in pre-column reactions [20]. It reacts with the thiolate anion in a thiol–disulfide exchange reaction, resulting in the formation of the yellow derivate. DTNB is widely utilized for the derivatization of GSH and GSSG in the classical spectrophotometric, enzymatic recycling or GSH-recycling assay developed by Tietze [34]. It is based on the reduction of GSSG to GSH with glutathione reductase in the presence of NADPH and formation of the colored product 5-thionitrobenzoate [19]. DTNB has been employed in several studies [35, 40, 41]. Alternative application of DTNB using the reagent for post-column reaction was shown by Nozal et al. [42]. A lot of evidence has accumulated that many protein sulfhydryls give an incomplete reaction with Ellman's reagent, even during prolonged assay times. Reiner et al. [43] solved this kinetic problem by including cystamine as a "mediator" between the protein sulfhydryl and Ellman's reagent.

Another representative of this group are 4, 4'-dithiodipyridine (DTDP) and 2, 2-ditiopyridine which reacts with thiols in an exchange reaction. DTDP is more sensitive and reactive thiol detection agent than DTNB and it can be used at lower pH (≥ 4.5 instead of at pH 8.0), however resistance to hydrolysis is lower [2, 19, 20]. Due to its small size, amphiphilic nature, and lack of charge, DTDP quickly reacts with poorly accessible protein sulfhydryls [43].

As a derivatization reagent for UV detection, 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT) is used for the determination of different forms of plasma thiols: cysteine, cysteinylglycine, glutathione and homocysteine. The CMQT-HPLC-UV method provides quantitative information on total, free and protein-bound thiols based on assays with derivatization after reduction of whole plasma or its acid-soluble and acid-precipitated fractions. Samples were reduced with sodium borohydride [44, 45] or tris (2-carboxyethyl) phosphine [29]. This reagent was also used for determination of glutathione and other thiols in urine [46] and saliva [47].

The derivatization reagents commonly used for determination of endogenous and exogenous thiols are: 2-halopyridinium and 2-haloquinolinium salts which react rapidly with thiols in slightly alkaline water solution to form stable S-pyridinium or

S-quinolinium derivatives. Reactions with these reagents enable multi thiol measurement within 1–15 min at room temperature [20].

Fluorescent thiol reagents

Derivatization with fluorescent thiol reagents is widely used in chromatographic or electrophoretic separation as a sensitive method for the detection of thiols. Fluorescent detection allows much lower detection limits than UV. Reagent must form GSH adducts with fluorescence yield to permit the measurement of GSH at picomolar amounts or less. Fluorophores that react with the thiol group are the most selective but they do not allow the detection of GSSG. Reagents targeting the amino group allow the simultaneous fluorimetric determination of GSH and GSSG, even if less selectively [19].

The benzofurazans: ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F), 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F) and 4-(*N*,*N*dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBDF) are representatives of this group. The activity of these reagents is in this order: DBD-F > ABD-F > SBD-F but both the selectivity to thiols and the solubility in aqueous solution are opposite: SBD-F > ABD-F > DBD-F [20]. The unreacted reagents and its hydrolysis products are non-fluorescent the thiol adducts are stable. Thus, they exhibit excellent sensitivity and specificity towards sulfhydryl, resulting in no interfering reagent peaks [19]. SBD-F is the most popular reagent in this group used in different analysis [9]. The drawbacks of using SBD-F are: a long derivatization time and high temperature requirements (1h 60°C). Thiol–SBD-F derivatives are stable for at least 8 h when protected from light [48]. In contrast, ABD-F offers fast and quantitative reaction under mild conditions, and is therefore suitable for reactions prior to CE [19]. Their advantage is that they do not cross-react with phosphines; thus, disulfide reduction and thiol derivatization can take place in the same step [2, 49, 50]. They have also much higher selectivity to thiols than bimane. The drawbacks are facts that benzofurazans react very slowly and that is why derivatization with this group requires drastic conditions (pH 9.5; 60°C for1h that are likely to pose a risk of reoxidation of GSH [2]. The derivatives with a benzofurazan structure are: 4-(*N*-acetylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole, 4-(*N* trichloroacetylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole and 7-chloro-*N*-[2-(dimethylamino) ethyl]-2,1,3-benzoxadiazole-4- sulfonamide [51].

Bimanes are found practical reagents which react rapidly with analyzed thiols. One example of this group is monobromobimane which reacts rapidly, but not specifically, with thiols at pH 8.0 at room temperature [48].

This reagent enables relatively high fluorescence emission and allows detection even in small concentration of analytes. The drawbacks are that the reagent itself and the hydrolysis products are fluorescent and undergoes photooxidation Moreover they co-react with thiol-containing reductants, other thiols and phosphines [2, 53]. It was used in determination of glutathione in plasma [29, 37] whole blood and saline [53] myoblasts in human multinucleated muscle fibers [54], red blood cells [55], mitochondria [56], human endothelial cells [57] and astrocytes [58].

Ortho-phthalaldehyde (OPA) can react with both the sulphydryl and amino group of thiols to form a highly fluorescence derivative [2]. OPA exhibits no native fluorescence, but during the reaction of OPA with a thiol generates a thiol-2-alkyl-substituted isoindole which is highly fluorescent. It is monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm. OPA reacts with thiols in thiol-selective and heterobifunctional reactions. In the thiol-selective OPA reaction this is necessary to use amine as a co-reagent prior to the derivatization reaction to achieve selective derivatization of thiols. Glycine, 2-aminoethanol, 2-mercaptoethanol, N-acetylcysteine, 3-mercaptopropionic acid or ethanethiol are commonly used as the co-reagents [19]. In the heterobifunctional reactions OPA reacts directly with the compounds containing both thiol and amine groups such as: GSH and γ-Glu-Cys without addition of the co-reagent [59]. However, co-reagents added to aminothiols direct the OPA reaction selectively towards thiols [19]. GSSG cannot react with OPA to form fluorescent derivative but after its hydrolysis at pH 12 it is possible to obtain fluorescent derivative [25]. Reactions with this reagent require mild reaction conditions and enable determination of some amino acids. However, simultaneous measurement of multiple aminothiols with OPA involves alkylation of free thiols. OPA also has the advantage of a short derivatization time at room temperature [39].

Optimal pH for derivatization with this reagent is between 9.5 and 12 [13, 60÷63].

The main disadvantages of reactions with OPA include that: the reactions are pH sensitive and the fluorescent thiol adducts are unstable in aqueous solution because of hydrolysis by excess OPA [59]. Naphthalene-2,3-dicarboxaldehyde, a reagent similar to OPA, has been successfully used for the determination of GSH levels [19, 27, 51].

Halides are IAA type reagents used for the determination of thiols in real samples. The analogues of IAA are also: 5-iodoacetamidofluorescein [65÷68] and 6-iodoacetamidofluorescein [69] containg fluorescein as fluorescent groups. The reagents were mainly used for the determination of thiols by capillary electrophoresis-laser induced fluorescence [51].

Conclusion

Sample preparation appears as the most important and the most complicated step at once in procedures of determination of glutathione and other thiols. This is connected with difficulties with removing sometimes complicated matrixes used for glutathione determination. Glutathione content in mammals has been analyzed generally in plasma, whole blood and erythrocytes. However those analytical methods are also helpful for determination amino thiols almost in every tissue and organ, what makes them more useful than enzymatic methods based on blood analysis commonly used in laboratories [70].

Sample preparation stages are similar for all chromatographic and electrophoretic methods. Every tissues and organs must be chilled after collecting and storage at low temperature from -20°C to -80 °C what depends on time from preparation to samples processing. Blood or plasma samples must be protected from coagulation by adding to fresh collected blood chelating reagents or placing blood in special tubes with chelator.

Deproteinization, reduction and derivatization procedures are not demand in every chromatographic or electrophoretic method. It also depends on kind of analyzed tissue or cell. Those with high concentration of glutathionylated proteins or γ-glutamyl transpeptidase must be deproteinized necessarily. Determination of total glutathione or quantification of disulfide bonds concentration requires reduction of disulfides. Derivatization step is necessary almost for all analytical methods described above, except for CE or tandem mass spectrometry what makes them less laborious and time-consuming than the other methods. Elimination of this step protect from undesirable derivatives: derivatizing reagent- other amino thiols, which are not determined.

Literature

1. Serru V., Baudin B., Ziegler F., David J.P., Cals M.J., Vaubourdolle M., MarioN: *Quantification of Reduced and Oxidized Glutathione in Whole Blood Samples by Capillary Electrophoresis.* Clin. Chem. 2001, 47, **7,**1321-1324.
2. Pastore A., Federici G., Bertini E., Piemonte F: *Analysis of glutathione: implication in redox and detoxification.* Clin. Chim. Acta. 2003, 333,**1,**19–39.
3. Bilska A., Kryczyk A., Włodek L.: *The different aspects of the biological role of glutathione.* Post. Hig. Med. Dośw. 2007, 61, **1,** 438-453.
4. Winiarska K.: *Glutation: niezwykłe funkcje pospolitego tripeptydu.* Post. Bioch. 2000, **46,** 318-326.
5. Radwańska-Wala B., Buszman E., Drużba D.: *Reactive oxygen species in pathogenesis of central nervous system diseases.* Wiad. Lek. 2008, 66, **1-3,** 67-72.
6. Tylec, A., Jarząb, A., Stryjecka- Zimmer, M., Wójcicka, A.: *Stress oxidative in schizophreni.* Pol. Merk. Lek. 2007, **23,** 133, 74-76.
7. Kałużna-Czaplińska, J., Grys, W., Szymańska, A., Rynkowski, J.: Role *of oxidative stress in autism.* Nowa Pediat. 2008**, 4,** 67-70.
8. Miller E., Mrowicka M., Zołyński K., Kedziora J.: *Oxidative stress in multiple sclerosis.* Pol. Merk. Lek. 2009, **27,** 162, 499-502.
9. Nolin T.D., McMenamin E., Himmelfarb J.: J. Chromatogr. B. 2007, **852**, 1-2, 554-561.
10. Sakhi A.K., Blomhoff R., Gundersen T.E.: *Simultaneous and trace determination of reduced and oxidized glutathione in minute plasma samples using dual mode fluorescence detection and column switching high performance liquid chromatography.* J. Chromatogr. A. 2007, **1142**, 2, 178-184.
11. Iwasaki Y., Saito Y., Nakano Y., Mochizuki K., Sakata O., Ito R., Saito K., Nakazawa H.: *Chromatographic and mass spectrometric analysis of glutathione in biological samples.* J. Chromatogr. B. 2009, **877,** 28, 3309–3317.
12. Overbo K., Sorbye H., Svardal A., Grong K., Svanes K.: *Glutathione and N-acetylcysteine reduce gastric mucosal blood flow in rats.* Dig. Dis. Sci. 1997 **42,** 8, 1765-1774.
13. Lenton K., Therriault H., Wagner J.R.: *Analysis of glutathione and glutathione disulfide in whole cells and mitochondria by postcolumn derivatization high-performance liquid chromatography with ortho-phthalaldehyde.* Anal. Biochem. 1997, **274,** 1, 125–130.
14. Hiraku Y., Murata M., Kawanishi S.: *Determination of intracellular glutathione and thiols by high performance liquid chromatography with a gold electrode at the femtomole level: comparison with a spectroscopic assay.* Biochim. Biophys. Acta. 2002, **1570,** 1, 47-52.
15. Lakritz J., Plopper C.G., Buckpitt A.R.: *Validated high-performance liquid chromatography-electrochemical method for determination of glutathione and glutathione disulfide in small tissue samples.* Anal. Biochem. 1997, **247**, 63–68.
16. Lazzarino G., Amorini A.M., Fazzina G., Vagnozzi R.,Signoretti S., Donzelli S., Di Stasio E., Giardina B., Tavazzi B.: *Single-sample preparation for simultaneous cellular redox and energy state determination*. Anal. Biochem. 2003, **322**(1), 51–59.
17. Potesil D., Petrlova J., Adam V., Vacek J., Klejdus B., Zehnalek J., Trnkova L., Havel L., Kizek R.: *Simultaneous femtomole determination of cysteine, reduced and oxidized glutathione, and phytochelatin in maize (Zea mays L.) kernels using high-performance liquid chromatography with electrochemical detection.* J. Chromatogr. A. 2007, **1084**(1), 134–144.
18. Mendoza J., Soto P., Ahumada I., Garrido T.: *Determination of oxidized and reduced glutathione, by capillary zone electrophoresis, in Brassica juncea plants treated with copper and cadmium.* Electrophoresis. 2004, **25**(6), 890-896.
19. Monostori P.,Wittmann, G., Karg E., Túri S.: *Determination of glutathione and glutathione disulfide in biological samples: An in-depth review.* J. Chromatogr. B. 2009, **877**, 3331–3346.
20. Kuśmierek K., Chwatko G., Głowacki R., Kubalczyk P., Bald E.: *Ultraviolet derivatization of low-molecular-mass thiols for high performance liquid chromatography and capillary electrophoresis analysis.* J. Chromatogr. B. 2011, **879**, 1290–1307.
21. Jones D.P.: *Glutathione measurement in human plasma. Evaluation of sample collection, storage and derivatization conditions for analysis of dansyl derivatives by HPLC.* Clin. Chim. Acta. 1998, **275**, 175–184.
22. Camera E., Picardo M.: *Analytical methods to investigate glutathione and related compounds in biological and pathological processes.* J. Chromatogr. B. 2002, **781**, 181–206.
23. McDermott G., Francis P.S., Holt K.J., Scott K.L., Martin S.D., Stupka N., Barnett M.W., Conlan X.: *Determination of intracellular glutathione and glutathione disulfide using high performance liquid chromatography with acidic potassium permanganate chemiluminescence detection.* Analyst. 2011, **136**, 2578-2585.
24. Harwood D.T., Kettle A., Brenna J.S., Winterbourn C.C.: *Simultaneous determination of reduced glutathione, glutathione disulphide and glutathione sulphonamide in cells and physiological fluids by isotope dilution liquid chromatography–tandem mass spectrometry.* J. Chromatogr. B. 2009. **877** (28), 3393-3399.
25. Kandár R., Žákováa P., Lotkováb H., Kučerab O., Červinková Z.: *Determination of reduced and oxidized glutathione in biological samples using liquid chromatography with fluorimetric detection.* J. Pharm. Biomed. Anal. 2007, **43**, 1382-1387.
26. Chang C., Tseng W.: *Gold nanoparticle extraction followed by capillary electrophoresis to determine the total, free, and protein-bound aminothiols in plasma.* Anal. Chem. 2010, **82** (7), 2696–2702.
27. Marchand S., Guzek A., Leroy P.: *HPLC study of the host–guest complexation between fluorescent glutathione derivatives and β-cyclodextrin.* J. Incl. Phenom. Macro. 2009, **66**(3-4), 409-418.
28. Hansen R.E., Østergaard R.,Nørgaard P., Winther J.R.: *Quantification of protein thiols and dithiols in the picomolar range using sodium borohydride and 4,4'-dithiodipyridine*. Anal. Biochem. 2007, **363**(1), 77-82.

29. Głowacki R., Bald E.: *Fully automated method for simultaneous determination of total cysteine, cysteinylglycine, glutathione and homocysteine in plasma by HPLC with UV absorbance detection. J. Chromatogr. B. 2009,* **877***(28)*, 3400-3404.
30. Hansen R.E., Winther J.R.: *An introduction to methods for analyzing thiols and disulfides: Reactions, reagents, and practical considerations.* Anal. Biochem. 2009, **394**(2), 147–158.
31. Michaelsen J.T., Dehnert S., Giustarini D., Beckmann B., Tsikas. D.: *HPLC analysis of human erythrocytic glutathione forms using OPA and N-acetylcysteine ethyl ester: Evidence for nitrite-induced GSH oxidation to GSSG.* J. Chromatogr. B. 2009, **877** (28), 3405–3417.
32. Jones D.P., Carlson J.L., Mody V.C., Cai J., Lynn M.J., Sternberg Jr. P.: *Redox State of Glutathione in Human Plasma.* Free Radic. Biol. Med. 2000, **28**(4), 625– 635.
33. Rosenfeld J.: Enhancement *of analysis by analytical derivatization.* J. Chromatogr. B. 2011, **879** (17-18), 1157–1158.
34. Tietze F.: *Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues.* Anal. Biochem. 1969, **27**(3), 502-522.
35. Rahman I., Kode A., Biswas S.K.: *Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method.* Nat. Protocols. 2007, **1**, 3159 – 3165.
36. Eyer P., Podhradský D.: *Evaluation of the micromethod for determination of glutathione using enzymatic cycling and Ellman's reagent.* Anal. Biochem. 1986, **153**(1), 5766.
37. Giustarini D., Milzani A., Dalle-Donne I., Rossi R.: *Red blood cells as a physiological source of glutathione for extracellular fluids. Blood Cells Mol. Dis.* 2008, **40**(2), 174-179.
38. Winters R.A., Żukowski J., Ercal N., Matthews R.H., Spitz D.R.: *Analysis of glutathione, glutathione disulfide, cysteine, homocysteine, and other biological thiols by high-performance liquid chromatography following derivatization by n-(1-pyrenyl)maleimide.* Anal. Biochem. 1995, **227**(1), 14-21.
39. Benkova B., Lozanov V., Ivanov I.P.,Todorova A., Milanov I., Mitev V.: *Determination of plasma aminothiols by high performance liquid chromatography after precolumn derivatization with N-(2-acridonyl)maleimide.* J. Chromatogr. B. 2008, **870** (1), 103–108.
40. Brehe J.E., Burch H.E.: *Enzymatic assay for glutathione. Anal. Biochem. 1976,* **74***(1)*, 189-97.
41. Katrusiak A.E., Paterson P.G., Kamencic H., Shoker A., Lyon A.W.: *Pre-column derivatization high-performance liquid chromatographic method for determination of cysteine, cysteinyl-glycine, homocysteine and glutathione in plasma and cell extracts.* J. Chromatogr. B: Biomedical Sciences and Applications. 2011, **758**(2), 207-2012.
42. Nozal M.J., Bernal J.L., Toribio L., Marinero P., Moral O., Manzanas L., Rodriguez E.: *Determination of glutathione, cysteine and N-acetylcysteine in rabbit eye tissues using high-performance liquid chromatography and post-column derivatization with 5,5'-dithiobis(2-nitrobenzoic acid).* J. Chromatogr. A 1997, **778**(1-2), 347-353.
43. Riener K., Kada G., Gruber H.J.: *Quick measurement of protein sulfhydryls with Ellman's reagent and with 4,4'-dithiodipyridine.* Anal. Bioanal. Chem. 2002, **373**(4-5), 266-276.
44. Bald E., Chwatko G., Głowacki R., Kuśmierek K.: *Analysis of plasma thiols by high-performance liquid chromatography with ultraviolet detection.* J. Chromatogr. A. 2004, **1032**(1-2), 109-115.
45. Iciek M., Chwatko G., Lorenc-Koci E., Bald E., Włodek L.*: Plasma levels of total, free and protein bound thiols as wellas sulfane sulfur in different age groups of rats.* Acta Biochim. Pol. 2004, **51**(3), 815–824.
46. Kuśmierek K., Głowacki R., Bald E.: *Analysis of urine for cysteine, cysteinylglycine, and homocysteine by high-performance liquid chromatography.* Anal. Bioanal. Chem. 2006, **385**(5), 855-860 .
47. Bald E., Głowacki R.*: Analysis of saliva for glutathione and metabolically related thiols by liquid chromatography with ultraviolet detection.* Amino Acids. 2005, **28**(4), 431-433.
48. McMenamin M.E., Himmelfarb J., Nolin T.: *Analysis of multiple aminothiols in human plasma by high performance liquid chromatography with fluorescence detection.* J. Chromatogr. B. 2009, **877**(28), 3274–3281.
49. Tang D., Wen L., Santschi P.: *Analysis of biogenic thiols in natural water samples by high-performance liquid chromatographic separation and fluorescence detection with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F).* Anal. Chim. Acta. 2000, **408**(1-2), 299-307.
50. Fermo I., Paroni R.: *Total plasma homocysteine analysis by HPLC with SBD-F precolumn derivatization.* Methods Mol. Biol. 2000,**159**, 237-244.
51. Toyo'oka T: *Analysis of thiols. J. Chromatogr. B.* 2009, 877 (28), 3318-3330.
52. Fujikawa Y., Urano Y., Komatsu T., Hanaoka K., Kojima H., Terai T., Inoue H., Nagano T.: *Design and Synthesis of Highly Sensitive Fluorogenic Substrates for Glutathione S-Transferase and Application for Activity Imaging in Living Cells.* J. Am. Chem. Soc. 2008, **130**, 14533–14543.
53. Wintner E.A., Deckwerth T.L., Langston W., Bengtsson A., Leviten D., Hill P., Insko M.A., Dumpit R., VandenEkart E., Toombs C.F., Szabo C.: *A monobromobimane-based assay to measure the pharmacokinetic profile of reactive sulphide species in blood.* Br. J. Pharm. 2010, **160**(4), 941–957.
54. Conlan X.A., Stupka N., McDermott G.P, Paul S. Francis P.S., Barnett N.W.: *Determination of intracellular glutathione and cysteine using HPLC with a monolithic column after derivatization with monobromobimane.* Biomed. Chromatogr. 2010, **24**(5), 455–457.
55. Sakhi A.K., Berg T.J.: *Reduced glutathione concentrations are not decreased in red blood cells of patients with long term type 1-diabetes.* Scand. J. Clin. Lab. Invest. 2011, **71**(2), 108-111.
56. Kimura Y., Goto Y., Kimura H.: *Hydrogen sulfide increases glutathione production and suppresses oxidative stress in mitochondria.* Antioxid. Redox Sign. 2010, **12**(1), 1-13.
57. Djurhuus R., Nossum V., Lundsett N., Hovin W., Svardal A., M., Havnes M.B., Fismen,L., Hjelde A., Brubak A.O.: *Simulated diving after heat stress potentiales the induction of heat shock protein 70 and elevates glutathione in human endothelial cells. Cell Stress Chaperones.*2010, **15**(4), 405-414.
58. Hilgier W., Węgrzynowicz M., Ruszkiewicz J., Oja S.S., Saransaari P., Albrecht J.: *Direct Exposure to Ammonia and Hyperammonemia Increase the Extracellular Accumulation and Degradation of Astroglia-Derived Glutathione in the Rat Prefrontal Cortex.* Toxicol. Sci. 2010, **117** (1), 163-168.
59. Zu Y.: *Molecular and Nanoparticle Postcolumn Reagents for Assay of Low-Molecular-Mass Biothiols using High-Performance Liquid Chromatography.* J. Chromatogr. B. 2009, **877**, 3358-3365.
60. Senft A.P., Dalton T.P., Shertzer H.G.: *Determining Glutathione and Glutathione Disulfide Using the Fluorescence Probe o-Phthalaldehyde.* Anal. Biochem. 2000, **280**, 80-86.
61. Cereser C., Guichard J., Drai J., Bannier E., Garcia I., Boget S., Parvaz P., Revol A.: *Quantitation of reduced and total glutathione at the femtomole level by high-performance liquid chromatography with fluorescence detection: application to red blood cells and cultured fibroblasts.* J. Chromatogr. B. 2001, **752**(1), 123-132.
62. Leroy P., Nicolas A., Wellmann M., Michelet F., Oster T., Siest G.: *Evaluation o-phthalaldehyde as bifunctional fluorogenic post-column reagent for glutathione in* LC. Chromatographia. 1993, **36**, 130-134.
63. Parmentier C., Leroy P., Wellman M., Nicolas A.: *Determination of cellular thiols and glutathione-related enzyme activities: versatility of high-performance liquid chromatography-spectrofluorimetric detection.* J. Chromatogr. B. 1998, **719**(1-2), 37-46.
64. Böhmer A., Jordan J., Tsikas D.: *High-performance liquid chromatography ultraviolet assay for humanerythrocytic catalase activity by measuring glutathione as o phthalaldehyde derivative.* Anal. Biochem. 2011, **410**(2), 296–303.
65. Musenga A., Mandrioli R., Bonifazi P., Kenndler E., Pompei A., Raggi M.A.: *Sensitive and selective determination of glutathione in probiotic bacteria by capillary electrophoresis-laser induced fluorescence.* Anal. Bioanal. Chem. 2007, **387**(3), 917-924.
66. Landino L.M., Moynihan K.L., Todd J.V., Kennett K.L.: *Modulation of the redox state of tubulin by the glutathione/glutaredoxin reductase system.* Biochem. Biophys. Res. Commun. 2004, **314**(2), 555-560.
67. Wang Y., Xie Y., Bernier M., Wainer I.W.: *Determination of free and protein-bound glutathione in HepG2 cells using capillary electrophoresis with laser-induced fluorescence detection.* J. Chromatogr. A. 2009, **1216** (16), 3533-3537.
68. Zinellu A., Sotgia S., Posadino A.M., Pasciu V., Zinellu E., Usai M.F., Scanu B., Chessa R., Gaspa L., Tadolini B., Deiana L., Carru C.: *Protein-bound glutathione measurement in cultured cells by CZE with LIF detection.* Electrophoresis. 2007, **28**(18), 3277–3283.

69. Caussé E., Malatray P., Calaf R., Charpiot P., Candito M., Bayle C., Valdiguié P., Salvayrel R., Couderc F.: *Plasma total homocysteine and other thiols analyzed by capillary electrophoresis/laser-induced fluorescence detection: Comparison with two other methods.* Electrophoresis. 2000, **28**(18), 3277–3283.
70. Araujo A., Saraiva, M., Lima, J.: *Determination of total and oxidized glutathione in human whole blood with a sequential injection analysis system.* Talanta. 2008, **74**(5), 1511-1519.

Ewelina BŁOŃSKA-SIKORA is a graduate of the Medical University of Lublin (Faculty of Pharmacy, 2008). Currently, she is a Ph.D. student of the Institute of Chemistry, Jan Kochanowski University in Kielce where she deals with the analysis of glutathione in human body. She has written 6 articles in the scientific press and she is the author or co-author of three posters at national and international conferences.

Tel.: 63317189, e-mail: ewelinablonska@wp.pl

Jerzy SZCZUDŁOWSKI – Ph.D., graduated from the Jagiellonian University. He is the author of 30 articles in international and Polish journals,, several dozen lectures and communications on domestic and foreign scientific conferences, . The Council Member of Editorial of the Aparatura Badawcza i Dydaktyczna journal. The member of Editorial in the Polish Normalization Committee. The reviewer applications in Operational Programme Innowacyjna Gospodarka. Currently, he performs function of the vice-director of Institute of Chemistry at Jan Kochanowski University in Kielce. The scientific practice: Sweden, University of Umea - Department of Public Health and Environmental Studies, Environmental Chemistry and National Defense Research The Establishment (1993)

Zygfryd WITKIEWICZ – Director of the Institute of Chemistry at Jan Kochanowski University in Kielce. He also works for Military Technical Academy in Warsaw. Professor is the chief editor of Aparatura Badawcza i Dydaktyczna journal, edited by COBRABiD. He is the president of Technical Committee responsible for Air Quality in Polish Normalization Committee and a member of The Analytical Chemistry Committee in Polish Academy of Science and a member of the Chromatographic Analysis commission, a member of PTChem, IUPAC and The Chromatographic Society. He published over 250 papers and 7 books. He is co-author of 21 patents. Professor received the Cwiet Medal granted by the Russian Chromatographic Association.

Dariusz WIDEŁ – M.Sc., graduated from the Mathematics and Sciences Faculty at Jan Kochanowski University of Humanities and Sciences in Kielce (chemistry, 2009). He works on the assistantship in Physical Chemistry Department at Institute of Chemistry, UJK. He is accomplishing the doctoral thesis ,, Application of electromigration and chromatographic method in waters and drinks analysis" under the direction of Prof. Zygfryd Witkiewicz.

International Symposium on Preparative and Industrial Chromatography and Allied Techniques (SPICA 2012)

30 September – 3 October 2012

Brussels, Belgium, Europe

Since 26 years, this Symposium is the event to attend to follow the state of the art in the field of Preparative and Industrial Chromatography. Workshops will be proposed on the opening day, Sunday September 30th where worldwide experts will share their experience to introduce and describe the main fields of Preparative Chromatography. During the symposium, the 3 days sessions will present the latest innovations as well as the industrial trends at development and production scales. The covered topics will range from process development to industrial applications of the purification techniques. The latest advances in process modelling and innovative processes (ie. multicolumn technologies) will be highlighted, keeping the regulatory, environmental and economic aspects into consideration. The evolution of the stationary phases will also be part of the program as well as the latest trends in membrane, extraction and other purification technologies.

Web Site: http://www.icsc2011.fr/