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The determination of hepatic glutathione at tissue and subcellular level

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ABSTRACT

Introduction: Glutathione (GSH) through its important function in the antioxidant protection of cells and in the conjugation of drugs and xenobiotics has crucial importance in pharmacology and toxicology. Since GSH is most often measured in liver tissue and different cell organelles it is important to choose the method that best suits for the determination of GSH.

Methods: The GSH content of cell organelles isolated from control and BSO-treated liver tissues was determined by the GSH-NEM-HPLC-UV, monochlorobimane-GSH-HPLC-fluorescence method and DTNB-GSH recycling assay to find the most suitable method for GSH determination from cell organelles.

Results: The GSH level of organelles could easily be measured by the monochlorobimane-HPLC-fluorescent method. The addition of monochlorobimane to the homogenisation buffer prevented the oxidation of GSH during isolation. The formation of monochlorobimane-GSH adduct was accelerated by the intrinsic GST activity of samples, however the omission of GST from the GSH standards could cause the overestimation of GSH content of biological samples. NEM is an excellent thiol protective agent and the GSH-NEM conjugate can be directly analysed by HPLC-UV, but the relatively high limit of detection made the method unsuitable for the determination of GSH from cell organelles. Although the DTNB-GSH recycling assay is quite simple and rapid the stabilization of GSH and the efficiency of detection lag behind the monochlorobimane-HPLC-fluorescent method.

Discussion: The monochlorobimane-HPLC-fluorescent method can be advised for the determination of GSH from pharmacologically and toxicological relevant cell organelles and liver tissue whilst addition of monochlorobimane to the homogenisation buffer prevented the autoxidation of GSH.

1. Introduction

Glutathione (GSH) is a water-soluble tripeptide composed of the amino acids glutamine, cysteine, and glycine. GSH is synthesized from its components in the cytosol where it is in the range of 1–10 mM (Meister, 1988). The biosynthetic pathway occurs virtually in all cell types, with the liver being the major producer and exporter of GSH. In most cells it can be found in millimolar concentration but in the hepatocytes it can reach about 10 mM (Forman, Zhang, & Rinna, 2009).

Glutathione plays multiple roles in the cells. The thiol group of GSH is a potent reducing agent. GSH is readily oxidized non-enzymatically to glutathione disulphide (GSSG) by electrophilic substances (e.g., free radicals and reactive oxygen/nitrogen species) (Yuan & Kaplowitz, 2009). GSH is a major determinant of intracellular redox potential. Because of its abundance and highly reduced state the GSH/GSSG ratio serves as a sensitive factor of cellular redox state (Yuan & Kaplowitz, 2009). In hepatic mitochondria where catalase is absent, glutathione peroxidase quenches H_2O_2 and repairs lipid oxidation by converting lipid hydroperoxides into less toxic alcohol derivatives (Yuan & Kaplowitz, 2009). All these reactions are fuelled by electrons from the reduced glutathione pool. GSH also scavenges reactive nitrogen species (RNS) such as peroxynitrite (ONOO) directly or indirectly with the help of glutathione peroxidase (Yuan & Kaplowitz, 2009). A further important function of GSH is to conjugate with drugs and xenobiotics under the catalysis of glutathione S-transferases (GSTs).

Extensive glutathione depletion is involved in different cell deaths such as apoptosis, necroptosis and autophagy (Lőrincz, Jemnitz, Kardon, Mandl, & Szarka, 2015; Mancilla et al., 2015; Plačková et al., 2016; Xie et al., 2015). Furthermore GSH and GSH depletion plays a crucial role in the recently described novel programmed cell death, ferroptosis (Dixon et al., 2012).

On the base of the above observations it can be unequivocally

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accepted that in toxicological studies the determination of GSH and GSSG in liver tissue and different cell organelles of liver cells such as mitochondria and endoplasmic reticulum has special importance.

A variety of spectrophotometric and HPLC methods are available to measure GSH (Fernández-Checa & Kaplowitz, 1990; Giustarini, Dalle-Donne, Milzani, Fanti, & Rossi, 2013; Giustarini et al., 2016; Griffith, 1980; Rice, Bump, Shrieve, Lee, & Kovacs, 1986; Tietze, 1969). The most critical point in the determination of GSH and GSSG is definitively the pre-analytical handling of the biological samples. The autoxidation of GSH to GSSG occurs during the pre-analytical sample preparation of many presently existing methods. It can cause up to 30–40% oxidation of GSH to GSSG and the most common source of analytical inaccuracy (Giustarini et al., 2016). Consequently, measured concentrations of GSH and GSSG vary considerably between laboratories that hinders the interpretation and comparison of different studies. The addition of the thiol masking agent N-ethylmaleimide (NEM) to the samples gives the possibility for accurate and precise estimation of GSH and GSSG (Giustarini et al., 2013; Tietze, 1969). The immediate addition of NEM to biological samples on one hand prevents the oxidation of GSH to GSSG, on the other hand the rapidly formed GSH-NEM conjugate can be analysed by reversed-phase HPLC with ultraviolet detection at 265 nm (Giustarini et al., 2013). Blood samples treated with NEM were stable at − 20 °C for 90 days (Giustarini et al., 2013). However this method is primarily suitable for the determination of GSH from samples with relatively high GSH content such as whole blood or isolated red blood cells. Unfortunately the relatively high limit of detection of the method makes it inappropriate in the case of samples with low GSH and GSSG levels such as plasma, other extracellular fluids or cell organelles.

Probably the most popular method is the GSH recycling assay (Tietze, 1969). It takes advantage of the specificity of the enzyme glutathione reductase (GR) to reduce GSSG to GSH in the presence of NADPH. The GSH content of the sample reacts with 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) to form the mixed disulphide GS-TNB and the chromophore 5-thio-2-nitrobenzoic acid (TNB). GS-TNB is reduced back subsequently to GSH by GR and NADPH or by direct reaction with any GSH still present in the assay mix. Therefore, instead of a single determination of how much DTNB reacts with GSH, the rate of TNB production is measured, as that is proportional to the initial amount of GSH. The method is generally applied to determine the total GSH (GSH + GSSG) concentration of the samples. The addition of a thiol masking agent such as NEM or vinylpyridine to the sample gives the possibility for the accurate and precise measurement of GSSG even at very low concentrations. Finally the concentration of GSH can be determined by subtracting the concentration of GSSG from that of the total GSH. The popularity of the method can be thanked to its rapid execution and relatively low cost.

A thiol probe that is freely permeable to plasma membranes and forms a fluorescent adduct specifically with GSH can greatly simplify and increase the sensitivity of the assay of cellular GSH. Kosower, Kosower, Newton, and Ranney (1979) found that bromobimanes are highly efficient labelling agents for cellular thiols. Following this observation monobromobimane (mBBr) became a widely used GSH labelling agent. Latter monochlorobimane (mBCl) was found to be less reactive with thiols than mBBr, forming a fluorescent adduct with GSH more specifically than mBBr (Fernández-Checa & Kaplowitz, 1990; Rice et al., 1986). mBCl was used as a sensitive and specific probe to analyse GSH in liver tissue, intact hepatocytes (Fernández-Checa & Kaplowitz, 1990; Lőrincz et al., 2015) as well as in different cell organelles (Nagy et al., 2010; Szarka, 2013).

In the present study we make an attempt to compare the above mentioned three methods to choose the one that is best suited for pharmacological and toxicological investigations. Since in toxicological, pharmacological studies, glutathione is most often measured in liver cells, tissues and different cell organelles of liver cells such as mitochondria and endoplasmic reticulum the concentration of GSH was determined from liver homogenate, mitochondrial, microsomal and cytoplasmic fractions isolated from liver of control and BSO treated male Wistar rats.

2. Materials and methods

2.1. Animal experiments

Male Wistar rats were purchased from Charles River (Gödöllő, Hungary). All animal experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, the European Communities Council Directive of 24 November 1986 (86/609/EEC). Animal treatments were approved by the Committee on Animal Experiments of Semmelweis University, Budapest. Rats were kept with ad libitum access to food and water. Experiments were undertaken when animals reached a body weight of 250–280 g. Rats were injected *i.p.* with BSO 700 mg/kg dissolved in PBS or with equal volume of PBS alone. Rats were sacrificed after 3 h of treatment.

2.2. Materials

Sucrose, HEPES, 5-sulfosalicylic acid, sodium phosphate, sodium hydroxide, EDTA, DTNB, Tris, N-ethylmaleimide, NADPH, reduced glutathione, glutathione reductase, glutathione-S-transferase were obtained from Sigma-Aldrich, St. Louis, MO. Monochlorobimane was purchased from Invitrogen. Trichloroacetic acid, acetic acid, methanol, acetonitrile were purchased from Reanal (Budapest, Hungary). The organic solvents used for HPLC were HPLC grade and the reagents used were of analytical grade.

2.3. Isolation of organelles from rat liver

The rat liver was homogenised using a Potter-Elvehjem homogeniser. Briefly a weighed amount of the tissue was put into the ice chilled glass tube containing cold isolation buffer. Three different isolation buffers were used: standard isolation buffer (300 mM sucrose, 20 mM HEPES pH 7.4, termed SH), standard isolation buffer supplemented with mClB (1 mM mClB, 300 mM sucrose, 20 mM HEPES pH 7.4, termed B-SH) and 5% w/V 5-sulfosalycilic acid. The latter isolation buffer was used for tissue homogenisation only, while the other two were used for organelle fractionation as well. The concentration of the primary homogenate was set to 20% w/V. The homogenisation was carried out on ice.

Fractionation of cell organelles was achieved by centrifugation. The isolation process was carried out on ice and in precooled devices as fast as possible to minimise decomposition of GSH.

The primary homogenate was centrifuged at 1000*g* 4 °C for 10 min to pellet cell nuclei and debris. The supernatant was used to determinate the GSH content of the liver homogenate. The supernatant was further centrifuged at 11,000*g* for 20 min at 4 °C to pellet mitochondria while the supernatant contained the microsomal fraction ant the cytoplasm. Separation of the latter ones was carried out by ultracentrifugation of the supernatant at 100,000*g* for 60 min at 4 °C. The previously pelleted mitochondrial fraction was washed with isolation buffer by centrifugation at 3000*g* for 10 min at 4 °C followed by pelleting at 11000 g for 20 min at 4 °C.

The mitochondrial and microsomal fractions were re-suspended in ice cold isolation buffer and together with the cytoplasmic fractions were used to determine GSH content of the given fraction.

2.4. Measurement of GSH by the DTNB method

The assay was based on the method of Baker, Cerniglia, and Zaman (1990). Briefly samples were diluted in DTNB assay buffer (100 mM sodium phosphate, 1 mM EDTA pH 7.5) to the appropriate concentrations.

50 µl of sample or GSH standard was measured on a microplate and 100 µl of DTNB reaction buffer was added (2.8 ml 1 mM DTNB, 3.75 ml 1 mM NADPH, 5.85 ml DTNB assay buffer, 20 U glutathione reductase). Absorption was monitored at 405 nm for at least 2 min to record the kinetics of TNB formation with a Thermo Scientific Multiskan GO microplate spectrophotometer. The absorption difference between 2 and 0 min was used for the quantification of GSH.

2.5. Measurement of GSH by mClB derivatisation and HPLC-fluorescent detection

Samples were diluted in Tris buffer (20 mM, pH 8.0) and mClB was rapidly added to a final concentration of 1 mM. The reaction was carried out at room temperature in dark and stopped after 15 min by adding TCA to a final concentration of 10% w/V. The conjugation reaction was also carried out in the presence of 100 mU/ml glutathione transferase.

To measure total glutathione, the samples were in prior treated with 1 mM DTT for 60 min in dark at room temperature.

Samples were centrifuged at 16,000*g*, 4 °C for 10 min to pellet precipitated proteins and 50 µl of the supernatant was loaded into a Waters 2690 HPLC for separation equipped with a Waters 2475 fluorescent detector set to 395 nm excitation and 477 nm emission wavelengths. Samples and standards were separated on a Teknokroma Nucleosil 100C-18, 5 µm, 250 × 4.6 mm column. A 40-min separation protocol was used employing the following linear gradients of 0.25% (v/v) aqueous acetic acid, NaOH, pH 3.5 (solvent A) and methanol (solvent B): 0 min at 15% (v/v) solvent B; 5 min at 15% (v/v) solvent B; 15 min at 23% (v/v) solvent B; 20 min at 23% (v/v) solvent B; 25 min at 100% (v/v) solvent B; 30 min at 15% (v/v) solvent B; 40 min at 15% (v/v) solvent B. The flow rate was 1 ml/min.

Based on standards, peaks with a retention time at 16 min were used for quantification of GSH.

2.6. Measurement of GSH by NEM derivatisation and HPLC-UV detection

This assay was based on the method of Giustarini et al. (2013) with minor modifications. Briefly 6 µl of 310 mM NEM was added to 500 µl of the samples and the reaction was carried out for 15 min in dark at room temperature then 80 µl of 100% w/V TCA was added. As the sensitivity of this assay was found to be underwhelming dilution of the sample was avoided by using concentrated TCA.

Samples were centrifuged at 16,000*g*, 4 °C for 10 min to pellet precipitated proteins and 50 µl was loaded into a Thermo Finnigan Surveyor HPLC equipped with Thermo Finnigan diode array detector. The absorbance spectrum between 220 and 350 nm was recorded.

Samples and standards were separated isocraticaly on a Teknokroma Mediterranean Sea C-18, 5 µm, 150 × 4.6 mm column with eluents A: 0.25% sodium acetate pH 3.1 and B: acetonitrile at a ratio of 94:6 respectively and at a flow rate of 1.25 ml/min.

Based on standards the first GS-NEM peak with a retention time of 7.5 min was used for GSH quantification which showed absorbance peak at 220 nm. This wavelength was used for GSH quantification. The second GS-NEM peak eluted at 9 min. Underivatised NEM had a retention time of 19.5 min and had characteristic secondary absorbance peak at 300 nm.

2.7. Measurement of the derivatisation kinetics of GSH by mClB

A Jasco FP-8200 spectrofluorometer was used to measure fluorescence at 385 nm of excitation wavelength and at 400–600 nm emission spectrum. The results were quantified at the peak emission wavelength which was found to be at 485 nm. The fluorescence intensity was recorded in 5 min intervals to avoid photobleaching. The reaction was carried out in a temperature controlled (23 °C) cuvette under continuous stirring. The used reaction buffer was 10 mM Tris pH 8.0 and mClB at a final concentration of 1 mM. In particular instances GST was also present at the indicated varying concentrations. The reaction was initiated by the addition of GSH to the cuvette at a final concentration of 1 µM.

2.8. Other methods

Protein quantities from samples were measured using Thermo Scientific BCA protein assay kit with a Thermo Scientific Multiskan GO microplate spectrophotometer according to the manufacturer's guidelines.

All data are expressed as means ± SD. Statistical analysis was carried out by using Student's *t*-test.

3. Results

3.1. The effect of GST: time course of mClB-GSH adduct formation

Different thiol masking agents can be applied to prevent the auto-oxidation of GSH. In our experiments we used monochlorobimane (mClB) as a fluorophore and a masking agent to inhibit the auto-oxidation of GSH during sample processing. Interestingly the application of GST was advised in the first FACS (Rice et al., 1986) and HPLC (Fernández-Checa & Kaplowitz, 1990) determinations of GSH by mClB to catalyse the formation of mClB-GSH adduct, but in other and later studies the enzyme was absent from the protocols (Bass, Ruddock, Klappa, & Freedman, 2004; Cotgreave & Moldéus, 1986; Fahey & Newton, 1987). Thus in the first set of our experiments the time course of the formation of mClB-GSH fluorescent adduct was investigated in the presence and absence of GST. In the absence of GST, GSH was only partially conjugated after 15smin of incubation and the reaction was not completed still after 45 min (Fig. 1). The addition of GST resulted in the significant acceleration of the reaction and it completed within 15 min (Fig. 1).

In the next step the effect of GST was investigated in the case of relevant biological samples. The addition of GST (in the same amount as earlier) enhanced the yield of bimane derived fluorescence of the mitochondrial samples and enhanced somewhat but not significantly

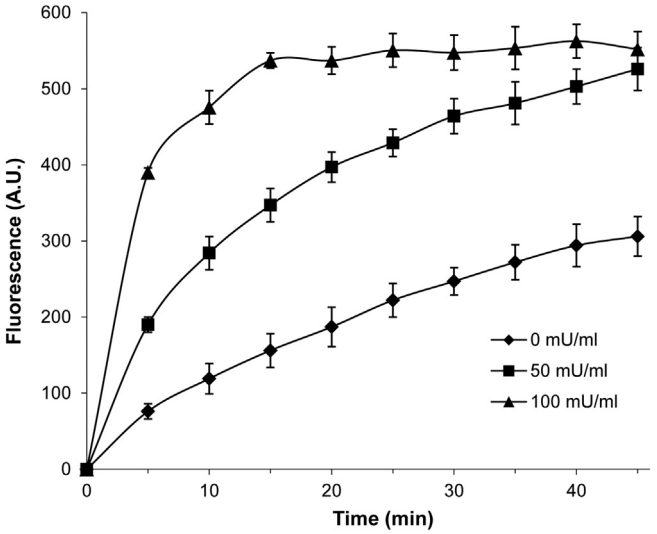

Fig. 1. The kinetics of the formation of the fluorescent bimane-GSH adduct in the presence or absence of GST. The reaction was carried out in a cuvette containing 20 mM Tris pH 8.0, 1 mM mClB and GST (if present) in the indicated concentration. The reaction was started by the addition of glutathione at the final concentration of 1 µM. The excitation wavelength of 385 nm was used while measuring the emission spectrum between 400 and 600 nm at 5 min intervals with a spectrofluorometer. The emission peak at 485 nm was used for quantification. All results are means ± standard deviation (SD) of three independent experiments.

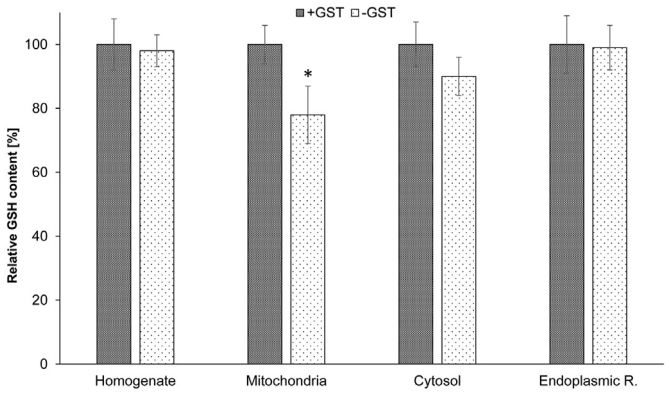

Fig. 2. The effect of GST addition on measurable bimane-GSH adduct formation in different cell organelles. Samples were used to determine GSH in the presence (+ GST) or absence (− GST) of additional 100 mU/ml GST by mClB derivatisation and HPLC-fluorescent detection as described in materials and methods. Results were normalised to samples in the given fraction containing additional GST (+ GST). All results are means ± standard deviation (SD) of three independent experiments. *Significantly different with respect to + GST ($P < 0.05$).

that of the cytosolic samples (Fig. 2) after 15 min of incubation interval. However it did not influence the bimane derived fluorescence of the microsomal samples (Fig. 2).

3.2. The investigation of the suitability of GSH-NEM-HPLC-UV method

To get closer to real pharmacological and toxicological problems and investigations rats were divided into two groups and treated with the inhibitor of γ-glutamyl-cysteinyl synthase BSO or with equal volume of vehicle (PBS). Tissue homogenate, mitochondrial, cytosolic and microsomal fractions were prepared from the liver and the GSH content was determined by the three different methods.

NEM is considered to be one of (or the) best thiol masking agents (Giustarini et al., 2016). Another advantage of the application of NEM is the fact that GSH-NEM conjugate is readily available for HPLC-UV detection (Giustarini et al., 2013). Unfortunately we found that the limit of detection for this method was very high. GSH standard with a concentration of 25 μM was the lowest that could reliably be quantitated. This relatively high limit of detection of the GSH-NEM-HPLC-UV method makes this method unsuitable for GSH determination from cell organelles, only samples from liver homogenate were measurable (Fig. 3).

3.3. The monochlorobimane-HPLC-fluorescent method is suitable for the determination of subcellular GSH content

Since mClB is nearly as good thiol masking agent as NEM (Fernández-Checa & Kaplowitz, 1990) we examined whether the addition of mClB prior tissue homogenisation prevents GSH auto-oxidation. BSO-treated and control rat livers were homogenised in sucrose-HEPES (SH) buffer or sucrose-HEPES buffer supplemented with 1 mM mClB (B-SH) and GSH was measured with the HPLC-fluorescent method. Total GSH was measured by pre-treating the samples with the reducing agent DTT as described in Materials and methods. Total GSH levels in liver homogenates from control animals isolated with either SH or B-SH buffers were similar (Fig. 4). GSH levels of liver homogenates from BSO-treated animals showed a marked decrease and were not significantly different isolated with either SH or B-SH. The addition of mClB to the isolation buffer (B-SH) had a significant effect on reduced GSH levels (Fig. 4). Interestingly the ratio of reduced GSH was higher in the samples from BSO treated animals (Fig. 4).

In order to investigate whether mClB can protect the GSH content of cell organelles from auto-oxidation liver homogenates in SH and B-SH buffers from control and BSO-treated animals were used for organelle

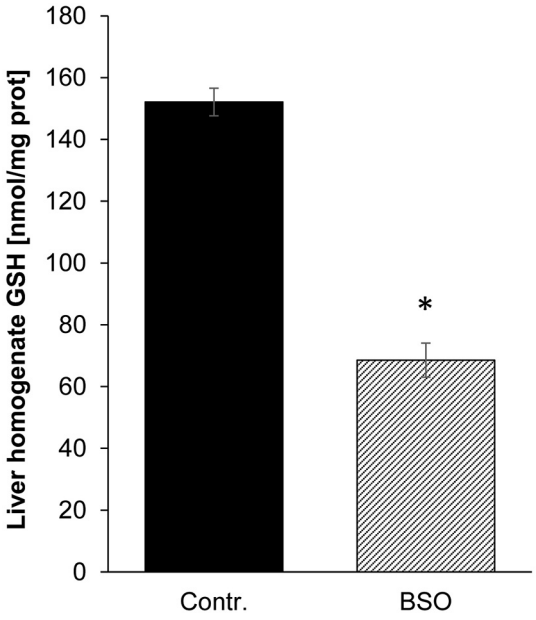

Fig. 3. Glutathione levels of control and BSO-treated rat liver homogenates measured by NEM-HPLC-UV method. Weighed tissue was homogenised in sucrose HEPES buffer and samples were conjugated with NEM as described. All results are means ± standard deviation (SD) of three independent experiments. *Significantly different with respect to control ($P < 0.05$).

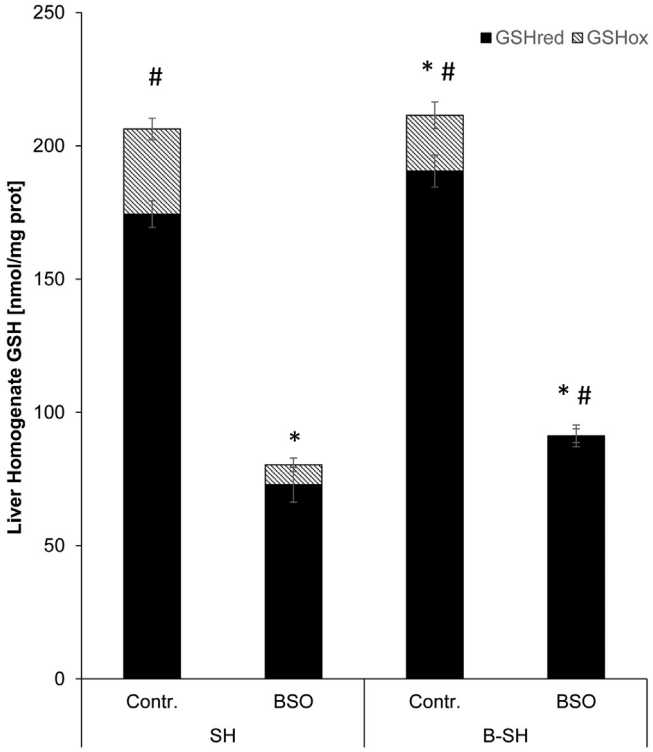

Fig. 4. Glutathione levels of control and BSO-treated rat liver homogenates measured by mClB-HPLC-Fluorescence method. The redox state of GSH was determined from control and BSO treated rat liver homogenates using mClB derivatisation and HPLC-fluorescent detection. The liver was either homogenised in sucrose-HEPES (SH) or sucrose-HEPES supplemented with 1 mM mClB (B-SH). Total GSH was measured by treating samples with 1 mM DTT for 60 min at room temperature in the dark. All results are means ± standard deviation (SD) of three independent experiments. The significance was assessed in the case of reduced GSH. *Significantly different with respect to control in Sucrose-Hepes buffer (SH) ($P < 0.05$). #Significantly different with respect to BSO treated in Sucrose-Hepes buffer (SH) ($P < 0.05$).

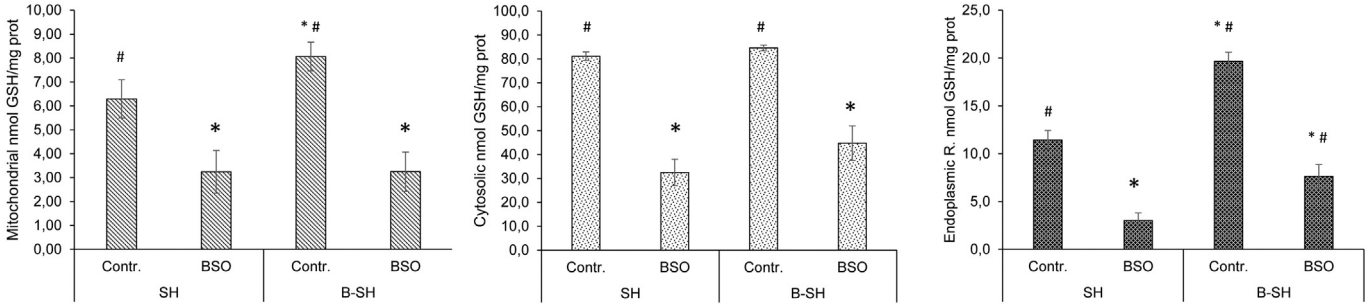

Fig. 5. GSH content of control and BSO treated rat liver fractions (mitochondrial, cytosolic and endoplasmic reticulum). GSH content of different fractions was determined using mClB derivatisation and HPLC-fluorescent detection. The liver was either homogenised in sucrose-HEPES (SH) or sucrose-HEPES supplemented with 1 mM mClB (B-SH). All results are means ± standard deviation (SD) of three independent experiments. *Significantly different with respect to control in Sucrose-Hepes buffer (SH) ($P < 0.05$). #Significantly different with respect to BSO treated in Sucrose-Hepes buffer (SH) ($P < 0.05$).

isolation. The presence of mClB in the homogenisation buffer resulted in elevated measurable reduced GSH levels in each fraction (Fig. 5). This protective effect of mClB became more significant by the time elapsed. More marked effects can be observed in the case of endoplasmic reticulum and cytosol fractions (Fig. 5).

3.4. The investigation of the suitability of DTNB-GSH recycling assay

The DTNB-GSH recycling assay takes the advantage of the specificity of glutathione reductase, it requires only a photometer it is rather quick and not so laborious. Thus we also investigated the suitability of this method for the determination of GSH from liver tissue homogenate and isolated cell organelles. The GSH content of samples can be stabilized by acidification thus the effect of 5% sulfosalicylic acid (SSA) as a homogenisation medium on measurable GSH levels was also studied. The homogenisation in 5% SSA indeed preserved somewhat the GSH content of the samples because samples homogenised in SH contained significantly lower measureable GSH levels with control being 8% and BSO-treated 19% less than the samples homogenised in 5% SSA (Fig. 6). However the homogenisation in 5% SSA makes the sample unsuitable for organelle fractionation and precipitates proteins. Hence measured homogenate GSH levels with the DTNB method had to be normalised to wet tissue weight. Thus GSH levels were also measured by the GSH recycling DTNB assay from fractioned organelles originating from liver samples homogenised in sucrose-HEPES buffer in order to compare the results achieved by different methods. In general we could measure lower GSH contents in each fractions than in the case of mClB-HPLC-fluorescent method (Fig. 5 vs Fig. 7).

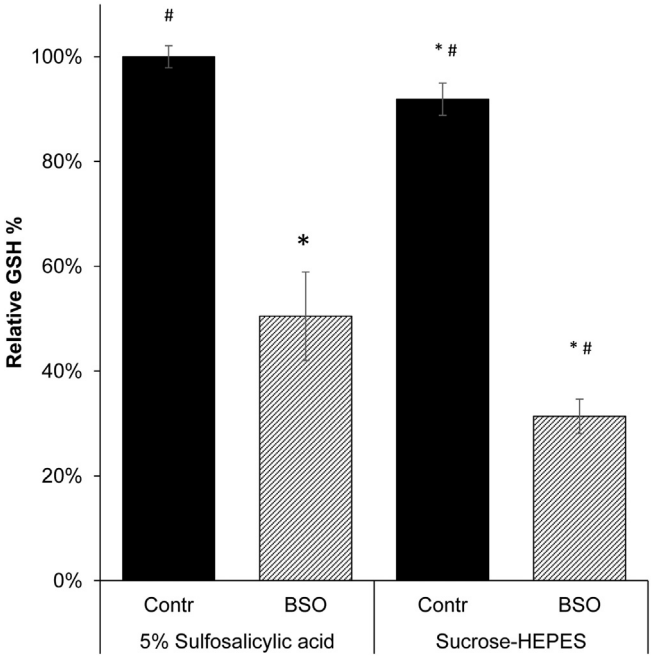

Fig. 6. Glutathione levels of control and BSO-treated rat liver homogenates measured by DTNB-GSH recycling assay. GSH was measured with DTNB-GSH recycling assay from rat liver homogenate homogenised in 5% sulfosalycilic acid (SSA) or Sucrose-HEPES (SH). Rats were either control or treated with BSO. Measured GSH was normalised to weighed wet tissue and to 5% SSA-control samples. All results are means ± standard deviation (SD) of three independent experiments. *Significantly different with respect to 5% SSA-control ($P < 0.05$). #Significantly different with respect to 5% SSA BSO-treated ($P < 0.05$).

4. Discussion

As it was stated earlier the crucial step of glutathione determination is the prevention of auto-oxidation of GSH. It can be accomplished by different thiol masking agents such as NEM, vinylpyridine and bimane (Fernández-Checa & Kaplowitz, 1990; Griffith, 1980; Rice et al., 1986; Tietze, 1969). Vinylpyridine is considered to be a slowly reacting masking agent (it reacts about 500-fold slower than NEM), furthermore it does not permeate cell membranes (Gorin, Martic, & Doughty, 1966; Lindorff-Larsen & Winther, 2000). Although the quick reaction of NEM with glutathione can prevent the oxidation of GSH and the formed GSH-NEM conjugate can be analysed by reversed-phase HPLC with ultraviolet detection (Giustarini et al., 2013), but the relatively high limit of detection of the GSH-NEM conjugate by HPLC-UV does not allow the determination of glutathione from samples with low GSH levels. The bimane derivative, monobromobimane (mBBr) was found to be somewhat slower than NEM (Giustarini et al., 2016), but it is sure that the conjugation of GSH with bimanes can be accelerated by the use of GST enzymes. Interestingly the application of GST was advised in the first FACS (Rice et al., 1986) and HPLC (Fernández-Checa & Kaplowitz, 1990) determinations of GSH by the more glutathione specific monochlorobimane, but in other and later studies the enzyme is absent from the protocols (Bass et al., 2004; Cotgreave & Moldéus, 1986; Fahey & Newton, 1987). In our experiments we also used mClB as a fluorophore and a masking agent to inhibit the auto-oxidation of GSH during sample processing. Thus in the first set of our experiments the time course of the formation of mClB-GSH fluorescent adduct was investigated in the presence and absence of GST. In the absence of GST, GSH was only partially conjugated after 15 min of incubation and the reaction was not completed still after 45 min (Fig. 1). The addition of GST resulted in the significant acceleration of the reaction and it played completely within 15 min (Fig. 1). Consequently the effect of GST was more than convincing in the case of pure GSH standard solutions. In the next step the effect of GST was investigated in the case of relevant biological samples. Thus tissue homogenate and pharmacologically and toxicological relevant cell organelles (mitochondria, cytosol and endoplasmic reticulum) were prepared from rat liver. The addition of GST (in the same amount as earlier) significantly enhanced the yield of bimane derived fluorescence of the mitochondrial samples and en-

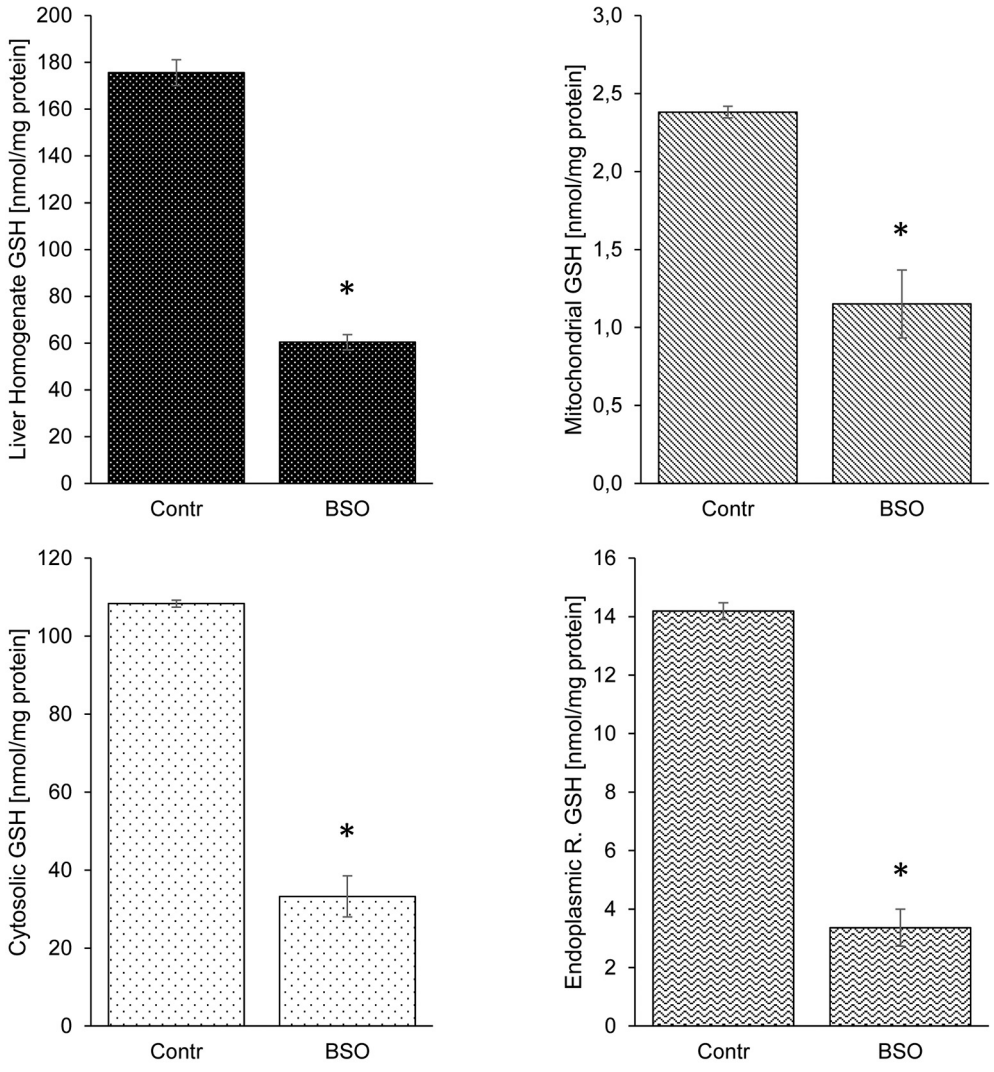

Fig. 7. Glutathione levels of fractioned rat liver organelles measured by DTNB-GSH recycling assay from BSO-treated and control animal tissues homogenised in sucrose-HEPES buffer. All results are means ± standard deviation (SD) of three independent experiments. *Significantly different with respect to control ($P < 0.05$).

hanced somewhat but not significantly that of the cytosolic samples (Fig. 2) after 15 min of incubation interval. However it did not influence the bimane derived fluorescence of the microsomal samples (Fig. 2). The addition of GST to the mitochondrial samples caused only a 22% elevation in the yield of mClB-GSH fluorescence (Fig. 2) that is much lower than it could be observed in the case of GSH standard solutions (Fig. 1). It is well known that all the investigated cell organelles contain GSTs which are structurally distinct and have separate evolutionary origins (Atkinson & Babbitt, 2009; Higgins & Hayes, 2011). Since all the organelles derived from liver tissue of control rats we can rule out the effect of different GST inducers (Higgins & Hayes, 2011). As it was seen earlier the effect of the lower amount of GST (50 mU/ml) lag behind the effect of higher amount (100 mU/ml) (Fig. 1). Unfortunately there is no quantitative data on the subcellular distribution of GSTs. However the preparation of cytosolic fraction accompanies by the significant dilution of the samples since it is the supernatant of the microsomal fraction. Although the cytosolic GSTs are relatively abundant proteins, but this dilution can easily cause that the level and activity of cytosolic and mitochondrial GSTs decrease under this critical limit during the isolation procedure.

The omission of GST from the standard GSH solutions cause the significant (approximately 2–3 fold) overestimation of GSH concentrations of biological samples. To demonstrate the difference the GSH concentrations of liver homogenates were calculated by standards with and without GST supplementation. The difference is really drastic (216.6 nmol/mg protein (with GST in the standards) vs. 307.5 nmol/mg protein (without GST in the standards) in the case of control liver homogenate).

As it was stated in the introduction the most critical point in the determination of GSH is the avoidance of autoxidation of GSH during the pre-analytical sample preparation that can be carried out by the immediate addition of different thiol masking agents such as NEM, vinylpyridine and bimane (Fernández-Checa & Kaplowitz, 1990; Griffith, 1980; Rice et al., 1986; Tietze, 1969). NEM is considered to be the one of (or the) best thiol masking agent (Giustarini et al., 2016). Beyond the covalent modification of oxidation-prone thiol group this method is readily available for UV detection of GSH-NEM conjugate (Giustarini et al., 2013). According to the earlier observations (Giustarini et al., 2013) we found that the limit of detection for this method was as high as 25 μM. To model the real pharmacological and toxicological problems and investigations rats were divided into two groups and treated with the inhibitor of γ-glutaimyl-cysteinyl synthase inhibitor BSO or with equal volume of vehicle (PBS). Tissue homogenate, mitochondrial, cytosolic and microsomal fractions were prepared from their liver and the GSH content of them was determined by the three different methods.

The relatively high limit of detection (25 μM) of GSH-NEM HPLC-UV method make the method unsuitable for GSH determination from

cell organelles, only samples from liver homogenate were measurable (Fig. 3). Homogenate samples had to be conjugated and measured without any dilution to keep samples in the range of quantification. It should again be noted that this method is considered to preserve mostly the GSH content of the samples (Giustarini et al., 2016). Because of this advantageous feature of the method it can use as a gold standard even if it is limited only to liver tissue homogenate samples.

Since mClB is nearly as good thiol masking agent as NEM (Fernández-Checa & Kaplowitz, 1990) we examined whether the addition of mClB prior tissue homogenisation prevents GSH auto-oxidation. BSO-treated and control rat livers were homogenised in sucrose-HEPES (SH) buffer or sucrose-HEPES buffer supplemented with mClB (B-SH). Total GSH levels in liver homogenates from control animals isolated with either SH or B-SH buffers were similar (Fig. 4). As it was expected total GSH levels of liver homogenates from BSO-treated animals showed a marked decrease and were not significantly different isolated with either SH or B-SH. However, we found that the addition of mClB to the isolation buffer (B-SH) had a significant effect on reduced GSH levels. The ratio of reduced GSH elevated from 80% to 90% in control and from 90% to almost 100% in BSO-treated liver homogenates due to mClB pre-treatment (Fig. 4). Since the ratio of reduced GSH in liver cells is around 99% (Yuan & Kaplowitz, 2009) these results indicate that mClB could (at least partly) prevent the oxidation of GSH to GSSG during the homogenisation. A further interesting observation could also be taken, the ratio of reduced GSH was higher in the samples from BSO treated animals (Fig. 4). It might be the result of a compensatory, more effective glutathione recycling.

The isolation of pharmacologically and toxicological relevant cell organelles such as mitochondria, cytosol and endoplasmic reticulum takes several hours thus the protective role of mClB can get more importance in the determination of GSH from these organelles. In order to investigate whether mClB can protect the samples from GSH auto-oxidation liver homogenates in SH and B-SH buffers from control and BSO-treated animals were used for organelle isolation. The presence of mClB in the homogenisation buffer resulted in elevated measurable reduced GSH levels in each fraction (Fig. 5). This protective effect of mClB became more significant by the time elapse. More marked effects can be observed in the case of endoplasmic reticulum and cytosol fractions (Fig. 5). The isolation of these fractions takes approximately two times longer than the isolation of mitochondrial one. These results reinforce that auto-oxidation results in significant loss of reduced GSH (Srivastava & Beutler, 1968) that can be prevented by the presence of mClB in the homogenisation buffer (from the beginning of the isolation of cell organelles). Furthermore this way the reduced GSH content of organelles can be determined much more precisely.

Not accidentally the most popular method for GSH determination is the GSH recycling assay (Tietze, 1969). It takes the advantage of the specificity of glutathione reductase, it requires only a photometer it is rather quick and not so laborious. Since the thiol group is much more stable than its ionized thiolate form the GSH content of samples can be stabilized by acidification. Unfortunately this acidification does not ensure full protection and the thiol group can also be oxidized during sample acidification (Giustarini et al., 2016). Thus we investigated the suitability of the DTNB-GSH recycling method for the determination of GSH from liver tissue homogenate and isolated cell organelles. Furthermore the effect of 5% sulfosalicylic acid (SSA) as a homogenisation medium on measurable GSH levels was also studied. We found that the homogenisation in 5% SSA indeed preserved somewhat the GSH content of the samples because samples homogenised in SH contained significantly lower measureable GSH levels with control being 8% and BSO-treated 19% less than the samples homogenised in 5% SSA (Fig. 6). However the homogenisation in 5% SSA makes the sample unsuitable for organelle fractionation and precipitates proteins. Thus GSH levels were also measured by the GSH recycling DTNB assay from fractioned organelles originating from liver samples homogenised in sucrose-HEPES buffer in order to compare the results achieved by different methods. The mClB-HPLC-fluorescent method resulted in higher total GSH values in each fractions than the GSH recycling DTNB assay (Fig. 5. vs Fig. 7). The difference between the two methods was even more significant when the fractions were isolated from liver tissue homogenised in mClB containing buffer (Fig. 5. vs Fig. 7). The marked difference can again be explained by the time elapsed during cell organelle isolation. The longer the required time for the isolation the more significant is the oxidation of GSH and more significant is the difference between the methods.

5. Conclusions

Our results underline the importance of the choice of method. First the sample has to be defined. After it can choose the most appropriate method for the determination of GSH. Although NEM is an excellent thiol protective agent and the formed GSH-NEM conjugate can directly be analysed by HPLC the relatively high limit of detection (25 μM) makes the method unsuitable for GSH determination from cell organelles. It can only be applied for the detection of GSH from tissue homogenates. The significantly lower GSH level of pharmacologically and toxicological relevant cell organelles could easily be measured by the mClB-HPLC-fluorescent method. Since the isolation of cell organelles takes several hours their GSH content can suffer from oxidation. The addition of mClB into the homogenisation buffer could prevent the oxidation of GSH to GSSG during the homogenisation and isolation procedure. An important point of this method is the preparation of standard solutions. The addition of GST resulted in the significant acceleration of the formation of mClB-GSH fluorescent adduct, its formation completed within 15 min. The formation of mClB-GSH fluorescent adduct in the biological samples is accelerated by their own GST activity, although it is also worth to pay attention to the possible limited GST activity of the biological samples too. The omission of GST from the standard GSH solutions can cause the significant (approximately 2–3 fold) overestimation of GSH concentrations of biological samples. Although the GSH recycling DTNB assay is quite simple and rapid the stabilization of GSH by SSA acidification can be characterised by lower efficiency than the formation of mClB-GSH fluorescent adduct. The application of NEM as a thiol masking agent inhibits the glutathione reductase the main enzyme of the detection (Griffith, 1980). The additional extraction of the excess NEM results in the loosing of its advantageous simplicity and quickness.

On the base of our experiments the following method can be recommended for the determination of GSH from pharmacologically and toxicological relevant cell organelles and liver tissue: the immediate addition of mClB to the homogenisation buffer stabilizes the GSH for the whole cell organelle isolation. The following HPLC-fluorescence detection of the forming mClB-GSH fluorescent adduct ensures the sensitive and automated determination of GSH.

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