Analytical Methods

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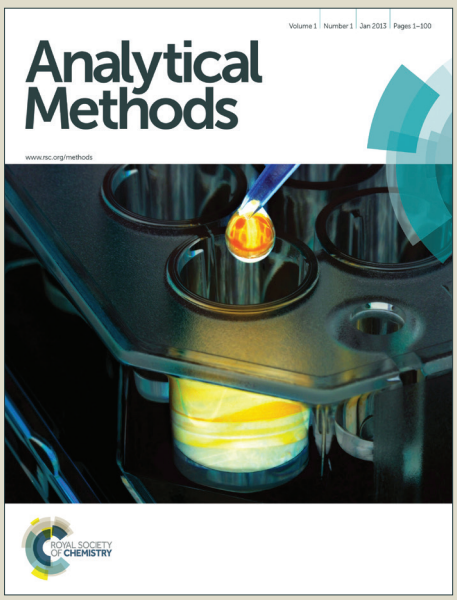

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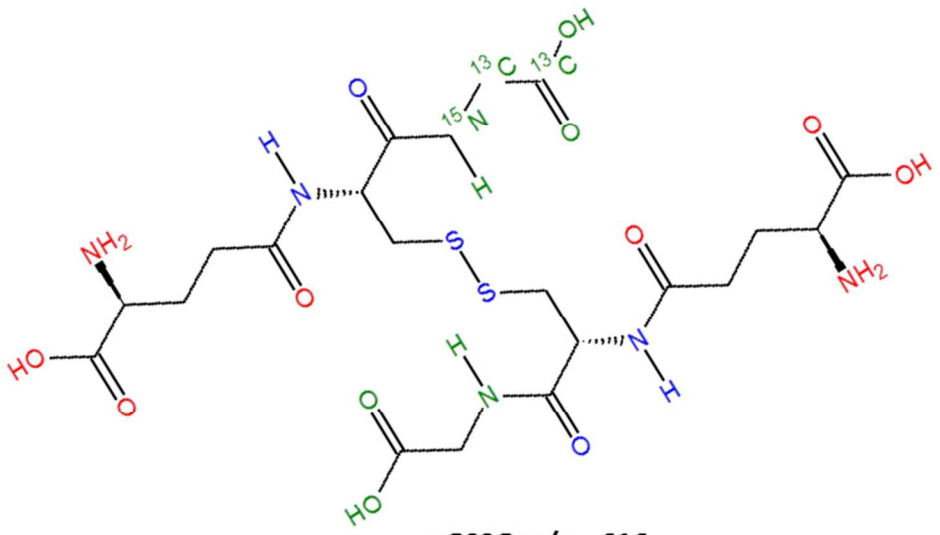

LC-MS/MS assays for accurate quantification of underivatized glutathione (GSH) and its oxidized form glutathione disulfide (GSSG), in cancer cell models, based on isotope dilution and the application in pre-clinical metallo-drug research.

Studying reduced versus oxidized glutathione in cancer cell models employing isotopically labelled standards

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Abstract

In this work, LC-MS/MS assays for accurate quantification of underivatized glutathione (GSH) and its oxidized form glutathione disulfide (GSSG) based on isotope dilution were developed. Both hydrophilic interaction- (HILIC) and reversed phase chromatography (RPC) were implemented. Different protocols dedicated to cancer cell lysis were validated in terms of extraction efficiency, recovery, and unwanted glutathione oxidation. The latter was monitored by isotopologues of GSSG, which were formed upon reaction with the isotopically enriched GSH and the natural GSH of the sample. Finally, LC-MS/MS was employed for studying the GSH:GSSG ratios in several cancer cells (HCT116, GLC4, SW480) upon exposure to anticancer metallodrugs. The clinically well-established cis-diamine-dichloro-platinum(II) (cisplatin) and sodiumtrans-[tetrachloridobis(1H-indazole)ruthenate(III)] (KP1339), a promising experimental drug were addressed. In both cases, a decrease of the GSH:GSSG ratio was observed upon drug exposure. It was more pronounced for cisplatin, where the ratio shifted from 440:1 to 240:1 and from 160:1 to 90:1 in HCT116 and GLC4 cells, respectively. For KP1339, a significant decrease was observed in the SW480 cancer cell model, whereas the change was not significant in HCT116 cells. Taken together this study introduces a new sensitive and robust method for the evaluation of drug-induced changes in intracellular GSH:GSSG ratio of human cells.

Introduction

Glutathione (y-glutamylcysteinylglycine) is a highly abundant thiol-containing pseudo tripeptide, present in tissue as well as body liquids. The chemistry of the corresponding redox couple glutathione (GSH) and its oxidized form glutathione disulfide (GSSG) [1,2] is essential in many biological processes including protein and DNA synthesis, metabolism as well as protection against free radical-induced damage [3,4,5]. The redox potential of the GSH:GSSG couple is an essential factor of cell functionality [6]. It is commonly accepted that the assessment of the molar ratio of GSH and GSSG provides valuable information about redox stress [7]. Thus, indications that a change in the ratio of GSH versus GSSG directly relates to redox stress are manifold [8,9,10,11,12,13,14,15,16,17,18,19,20,21]. Depending on the cell type, the molar GSH:GSSG ratio can exceed 100:1 up to 1000:1 [22,23,24]. Accordingly, oxidation of GSH will lead to a detrimental overestimation of GSSG [25,26,27,28,29,30,31]. This fact makes accurate assessment of the ratio analytically challenging.

Hence, the elaboration of quick and reliable sample preparation procedures e.g. providing high efficiency extractions and most importantly avoiding oxidation was and still is a major issue in quantitative analysis of the redox-sensitive GSH:GSSG couple, regardless which measurement technique is used. The state of the art regarding analysis in different target organisms and biological compartments as well as the implied analytical challenges have been comprehensively reviewed elsewhere [32,33,34]. For the analysis of cell models, i.e. addressing intracellular concentration levels, different extraction approaches utilizing a wide range of solvents were investigated in the past. Hot water, boiling ethanol [35], hot phosphoric acid, hot 5-sulfosalicylic acid [36], freeze thaw in methanol [37], and perchloric acid [38,39] are just a selection of lysis and extraction solvents used in literature. Practical considerations and investigation by Akerboom et al. (1981) and Anderson (1985) were suggesting that quenching and extraction with acidic solvents was the method of choice [40,36]. The benefits were (1) inactivation of enzymes which have glutathione as substrate, (2) the denaturation of proteins which are possible binding partners for GSH and their subsequent removal by centrifugation and the general decrease in reaction rate constants resulting in slowing-down of GSH oxidizing to GSSG. Chemical derivatization of GSH, e.g. alkylation with N-ethylmaleimide [33] was also successfully applied to obtain *in-vivo* snap shots like concentration levels. Evidently, the choice of appropriate derivatization strategy

directly relates to the choice of detection method and/or the chromatographic separation. Accordingly, a wide range of chromatographic separations have been established for the analysis of glutathione in its reduced and its oxidized form, based on reversed phase chromatography (RPC) [41,42,43], separations on pentafluorophenyl phases (PFP) [44], adsorption chromatography [27,45], hydrophilic interaction chromatography (HILIC) [42,46,47,48] and mixed mode [29,49] methods. Separation methods and detection methods for derivatized and non-derivatized glutathione compounds have been reviewed elsewhere [32].

Recently, we elaborated accurate assays addressing the GSH:GSSG ratio in the yeast *Pichia pastoris* without need of tedious derivatization[50]. Comparable to other studies [27,41,45,49], isotopically labelled standards have been implemented in this study for calibration. The isotopically enriched calibrant of GSSG was in-house synthesized. The scientific novelty consisted in the fact that isotopologues of GSSG generated during sample preparation were monitored, enabling the investigation of unwanted oxidation during the sample preparation procedures. **[Fig. 1]** shows the concept by depicting the oxidation product denoted as mixed oxidised glutathione (mGSSG) formed by reaction of the non-labelled GSH present in the sample and the isotopically labelled (spike added prior to sample preparation) GSH. Only using this monitoring tool, suitable procedures targeting GSH:GSSG ratios could be obtained leading to accurate values, despite the fact that no derivatization was applied. In this work, we validated sample preparation strategies dedicated to cancer cell models using this monitoring concept. Moreover, we improved the LC-MS methodological tool set by introduction of a new reversed phase separation method.

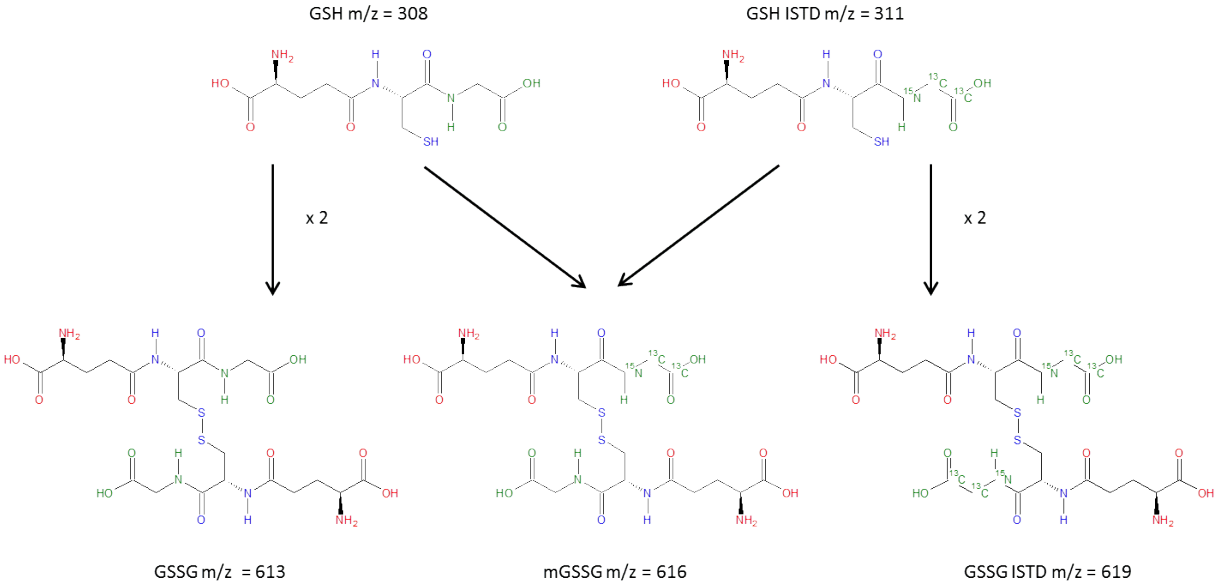

Fig. 1: Possible products of GSH m/z = 308 and its ($^{13}C_2$, ^{15}N-labelled glycine) labelled form $^{13}C_2$,^{15}N-GSH m/z=311. Differently labelled forms of GSSG as product of oxidation: GSSG m/z=613 as product of the monoisotopic GSH m/z = 308, $^{13}C_4$,$^{15}N_2$-GSSG m/z = 619 as product of the synthesis out of two $^{13}C_2$,^{15}N-GSH m/z = 311 and mGSSG m/z = 616 as product of GSH m/z = 308 and $^{13}C_2$,^{15}N-GSH m/z = 311 utilized to monitor oxidation.

Ever, since the introduction of the first metallodrugs in anticancer therapy, the role of glutathione as potential intracellular interaction partner has been investigated. In this work, the evaluated mass spectrometric assays enabled the investigation of this aspect in preclinical studies using cancer cell models. The methods were applied for studying the impact of the drugs on the total glutathione levels and the GSH:GSSG ratio, as important parameter for oxidative stress. Cisplatin, a clinically well-established anticancer drug and KP1339 [51,52,53], an investigational anticancer drug, were studied in different cancer cell lines. Notably, several studies have already indicated that resistance to cisplatin is frequently related to increasing levels of glutathione [54,55], while the impact of the intracellular glutathione levels with regard to KP1339 is still not fully evaluated [56]. Notably, the proposed mechanism of glutathione-related resistance against platinum drugs was so far exclusively based on an increase of intracellular glutathione pools and subsequent scavenging of cisplatin accompanied by cisplatin-GSH-adducts [57,58,59,60]. Undoubtedly, according to the hard soft acid base (HSAB) concept, cisplatin will have an higher affinity to the thiol groups of GSH than to the nitrogen donors of the DNA [61,62]. However, it has to be kept in mind that every free thiol group in

the cytosol will have the similar affinity to cisplatin and, therefore, the intracellular fate of cisplatin might be more complex than believed. This is also supported by a recent publication of our group addressing the reaction kinetics of cisplatin in the cell revealing that at physiological concentrations intact cisplatin (and not its GSH-adduct) was the predominant species in the low molecular weight fraction of the cytosol [63]. Consequently, aim of the here presented work was to investigate whether the resistance against platinum drugs based on increase of intracellular glutathione levels could also be related to enhanced stability of the redox balance.

Experimental

Reagents and standards

Reverse osmosis water was further purified before use (Ultra Clear basic Reinstwassersystem, SG Wasseraufbereitung und Regenerierstation GmbH, Barsbüttel, Germany). Acetonitrile (LC-MS grade) was obtained from Fluka, Buchs, Switzerland or from Fisher Scientific, Loughborough, Leicestershire, UK. Glutathione (≥ 98%), glutathione disulfide (~ 98%), glutathione-glycine-$^{13}C_2$,^{15}N-GSH, sodium iodide p.a. (≥ 99%), hydrogen peroxide (30% v/v), and Triton X-100 reduced were purchased from Sigma Aldrich, Vienna, Austria. Ortho-phosphoric acid (85% v/v), sodium chloride p.a. and formic acid (suprapur) were purchased from Merck, Darmstadt, Germany. Ethanol (99.5% v/v) and methanol (≥ 99% v/v) were purchased from Roth, Karlsruhe, Germany. KP1339 and cisplatin have been synthesized at the Institute of Inorganic Chemistry, Vienna, Austria according to previously published procedures [64].

Synthesis of $^{13}C_4$,$^{15}N_2$-GSSG

For the synthesis of the internal standard of GSSG a well-established and validated protocol was applied [65],[43],[50]. Briefly, 1 mg NaI was added to 1 mL of aqueous GSH-(glycine-$^{13}C_2$,^{15}N) solution (c = 6.5 mmol L^{-1}) resulting in a concentration of 0.67 mmol L^{-1}. This was followed by addition of 2 µL 30% hydrogen peroxide (resulting concentration 20 mmol L^{-1}) and heating at 50 °C for 2 h. Finally, the residual hydrogen peroxide was destroyed by heating at 65 °C for 5 min.

Separation via HILIC- and RPC

One method based on HILIC and one based on reversed phase chromatography were developed in course of this study. The separations were carried out on a LC system consisting of a Thermo Scientific CTC PAL autosampler and a Thermo Scientific Accela 1259 pump. The HILIC has a flow rate of 200 µl min^{-1} and an analysis time of 15 min. For separation a 150 x 2.1 mm ZIC-HILIC column (3.5 µm particle size) equipped with a 20 x 2.1 mm ZIC-HILIC guard column (5 µm particle size) from Merck (Darmstadt, Germany) was used. An injection volume of 5 µL was applied. Column temperature was set to 45 °C. The RPC separation was performed on an Atlantis T3® analytical column (150 x 2.1mm, 3 µm particle size, 100 Å pore size) from Waters (Milford, USA) equipped with an Atlantis T3 guard column (20 x 2.1, 3µm particle size) from Waters (Milford, USA). The column temperature for this approach was 40 °C. Injection volume was ass well 5 µl. The used gradients can be reviewed below **[Tab 1.]**.

HILIC:			**RPC:**		
Time (min)	**A %**	**B %**	**Time (min)**	**A %**	**B %**
0	40	60	2	100	0
6	90	10	10	70	30
7	90	10	12	0	100
7.1	40	60	12.1	100	0
15	40	60	17	100	0
A: 98.9% Water 1% ACN 0.1% HCOOH **B**: 98.9% ACN 1% Water 0.1% HCOOH			**A**: 99.9% Water 0.1% HCOOH **B**: 99.9% MeOH 0.1% HCOOH		
Flow rate: 200 µL min^{-1}			Flow rate: 250 µL min^{-1}		

Tab. 1: Gradient conditions of the two chromatographic methods applied in the study.

ESI-MS/MS analysis

Tandem mass spectrometric analysis was carried out on the Thermo Scientific TSQ Vantage MS/MS. Capillary temperature was 300 °C, vaporizer temperature was 350 °C. The collision gas pressure was 1.5 mTorr. The following settings were applied for SRM:

Precursor m/z	Product m/z	CE	Name
308.0	162.1	16	GSH
311.0	165.1	16	$^{13}C_2$,^{15}N-GSH
613.0	355.1	20	GSSG
616.0	358.1	20	mGSSG
619.0	361.1	20	$^{13}C_4$,$^{15}N_2$-GSSG

Tab. 2: Precursor and product ions for GSH and GSSG and their corresponding isotopically labelled standards for ESI-MS/MS analysis

Cell culture and preparation of cytosolic fractions

The colon carcinoma cell line SW480 has been purchased from the American Tissue Collection Center (ATCC) and is grown in minimal essential medium (MEM). The colon carcinoma cell model HCT116 was a gift from B. Vogelstein (John Hopkins University, Baltimore, USA) and grown in McCoy's medium. The small cell lung carcinoma cell line GLC4 is from E.G. deVries (University Hospital Groningen, Groningen, the Netherlands) and grown in RPMI-1640 medium. All media were supplemented with 10% fetal calf serum without antibiotics. Cultures were regularly checked for Mycoplasma contamination. These cell models represent types of cancer, where platinum-based therapy is also used in the clinical practice [66].

Cells ($1*10^6$) were seeded in a T25 cm^2 culture flask and allowed to attach for 24 h. Drugs (5 µM cisplatin, 100 µM KP1339) were added in 5 mL of fresh growth medium. After the indicated drug incubation cells were collected by scratching and washed with ice-cold PBS. The suspension was then centrifuged for 5 min at 1100 rpm and the supernatant discarded.

For cell lysis three protocols for the extraction of GSH and GSSG were validated regarding extraction efficiency and their impact on the GSH:GSSG ratios. During and between every step the samples were kept on ice. The internal standard was added to the extraction solvents before lysis of the cell pellets in concentrations that resemble natural occurring levels. The spiking concentration for $^{13}C_2$,^{15}N-GSH was 5000 µg L^{-1} (16.3 µM) and for $^{13}C_4$,$^{15}N_2$-GSSG 50 µg L^{-1} (82 nM).

Method 1 - Cold acidic extraction with H_3PO_4**:** The cell pellet was resuspended in 100 µL 0.1 M H_3PO_4 in an Eppendorf-tube, vortexed for 15 sec, and transferred into liquid nitrogen for 1 min then thawed at 20 °C and centrifuged for 1 min at 5000 rpm. The supernatant was collected into another Eppendorf-tube. The remaining pellet was again mixed with 100 µL 0.1 M H_3PO_4, vortexed, frozen, thawed, centrifuged, and collected. This procedure was repeated a third time resulting in 300 µL cytosolic extract which was finally centrifuged for 3 min at 14000 rpm and the supernatant transferred into a fresh Eppendorf-tube.

Method 2 - Cold acidic extraction with HCOOH: The cell pellet was resuspended in 100 µL 0.1% HCOOH in an Eppendorf-tube, vortexed for 15 sec, and transferred into liquid nitrogen for 1 min then thawed at 20 °C and centrifuged for 1 min at 5000 rpm. The supernatant was collected into another Eppendorf-tube. The remaining pellet was again mixed with 100 µL 0.1 % HCOOH, vortexed, frozen, thawed, centrifuged, and collected. This procedure was repeated a third time resulting in 300 µL cytosolic extract which was finally centrifuged for 3 min at 14000 rpm and the supernatant transferred into a fresh Eppendorf-tube.

Method 3 - Extraction at ambient temperature: The cell-pellet was diluted in 300 µL 0.1% Triton-X with 15 mM NaCl and vortexed for 1 min. Subsequently, the sample was centrifuged for 5 min at 14000 rpm. The supernatant was then transferred into a fresh Eppendorf-tube.

Regardless of the lysis protocol used, the undiluted cytosolic digestions were transferred into HPLC-vials, which were placed into the cooled (4 °C) autosampler of the HPLC.

Additional instrumentation

Between every step of the lysis protocols the samples were kept on ice, additionally the centrifugation steps have been performed with a cooled centrifuge. Cooled centrifugation (4 °C) was performed with a Mikro 200 R (Hettich Zentrifugen; 2424B rotor).

Results and discussion

LC-MS/MS analysis of GSH and GSSG

First, a novel reversed phase separation method was developed for the MS-based analysis of underivatized GSH and GSSG and compared to HILIC. The latter had been successfully implemented in a previous work of our group, dedicated to the analysis in yeast [50]. As a drawback, HILIC separations are known to require long re-equilibration times, in order to obtain high repeatability precision and robustness against biological matrixes. This may cause problems when unstable compounds (as GSH) need to be analysed in long measurement sequences or, in more general terms, this is the limiting factor regarding high-throughput analysis. In this work the overall analytical run time of the already available HILIC method could be significantly reduced to 15 min, compared to 19 min of our previous work [50], maintaining the necessary long re-equilibration time of 7.9 min. As can be readily seen in **[Tab. 3]**, the newly established reversed phase chromatography was superior to HILIC in terms of separation efficiency. A capacity factor of 1.4 for GSH was excellent considering the poor retention often found for this molecule on reversed phase stationary phases. Hence, this separation method was selected for the following investigation of cancer cell models. **[Tab. 4]** gives the analytical figures of merit obtained by LC-MS/MS determination. As can be observed, excellent limits of detection in the sub-nM range could be achieved. The overall instrumental precision (repeatability precision of peak areas) was 1.2% in the case of GSH and 1.4% for GSSG (n = 4).

	HILIC:		RPC:	
	GSH	GSSG	GSH	GSSG
Total run time / min	15		17	
Re-equilibration time /min	7.9		4.9	
Retention time / min	3.3	5	4	6.7
Peak width* / min	0.8	1	0.45	0.3
Capacity factor	1.1	2.2	1.4	3
Efficiency / N_{column}	270	400	4000	12000
HETP µm	550	380	37	13
Resolution GSH:GSSG	2		7	

*Peak width assessed at the baseline

Tab. 3: Figures of merit for the separation of GSH and GSSG for the applied chromatographic separations.

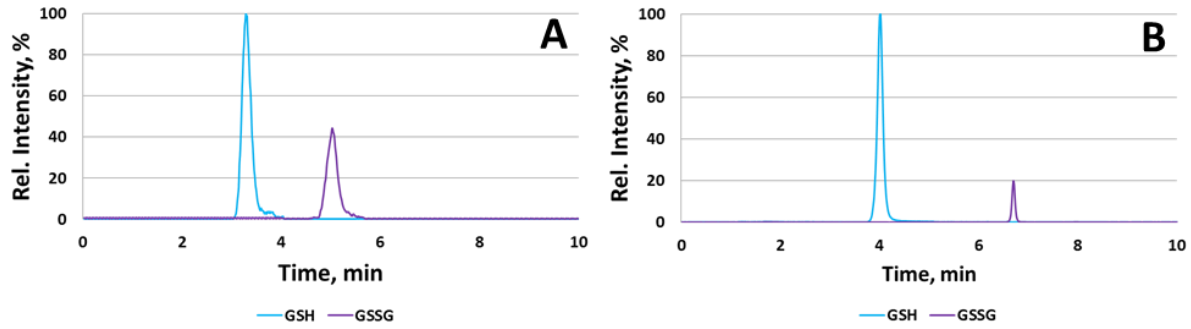

Fig. 2: **Fig. 2 A** HILIC separation of a 500 µg L^{-1} standard mix, GSH (1628 nmol L^{-1}) and GSSG (817 nmol L^{-1}), measured via LC- ESI-MS/MS in a 0.1 M H3PO4 solution. **Fig. 2 B** RPC separation of a standard mix, 1000 µg L^{-1} GSH (3256 nmol L^{-1}) and 500 µg L^{-1} GSSG (817 nmol L^{-1}), measured via LC- ESI-MS/MS in a 0.1 M H_3PO_4 solution.

Compound	LOD (3s) µg L^{-1}	LOQ (10s) µg L^{-1}	LOD nmol L^{-1}	LOQ nmol L^{-1}
GSH	1	2	2	8
GSSG	0.03	0.1	0.06	0.2

Tab. 4: Figures of merit for the detection of GSH and GSSG for the RPC separation in combination with tandem mass spectrometric detection

Evaluation of the employed lysis protocols

Sample preparation is the most critical step when addressing the accurate quantification of intracellular GSH and GSSG. Both, extraction efficiency and recovery need to be considered implementing isotope dilution for both compounds. While for GSSG the extraction efficiency is the most critical parameter, for GSH special emphasis is on the extraction recovery when establishing sample preparation procedures. In previous studies, complete disintegration of yeast cell wall was shown to be a prerequisite for quantitative analysis of GSSG [50]. Lysis protocols resulting only in permeabilization of the cell wall showed significantly lower extraction efficiencies [50]. However, this was not surprising considering the size of the pseudopeptide GSSG. In this study focusing on mammalian cells, whose cell membrane is evidently less stable than yeast cell walls and, hence, significantly easier to lyse, the tested sample preparation procedures involved rather mild chemical conditions at reduced temperatures. The GSH:GSSG ratio in cancer cells was expected to be rather low (in literature 1:100-1:1000 [22,23,24]) compared to yeast cells, where average cytosolic ratios of 1:20 are typical [67,68]. Accordingly, not only recovery and extraction yield were carefully investigated for different cell lysis methods, but also unwanted oxidation of GSH resulting in elevated yield of GSSG was considered.

At this point it has to be noted that quenching of the cellular metabolism was not considered prior to extraction since it could be shown in yeast samples that this step could be omitted. This could be explained by the large pool size of the investigated glutathione and the low turnover rates compared to other primary metabolites (e.g. sugar phosphates). Nevertheless, the time of scraping the cells in ice cold PBS was kept as short as possible.

Three lysis methods were investigated more closely. On the one hand, a lysis protocol utilizing the detergent Triton X (1), a common procedure for cytosol preparation of mammalian cells e.g. for Western blotting, was employed. On the other hand, two acidic freeze thaw approaches based on 0.1 M H_3PO_4 (2) and 0.1% HCOOH (3) were evaluated.

In a first step, standard mixtures were processed according to the 3 lysis protocols. The concentration levels used in these cell free - validation experiments resembled the typical large excess of GSH versus GSSG present in mammalian cell preparations (containing 16.3 µM GSH, 16.3 µM $^{13}C_2$,^{15}N-GSH, 16.3 nM GSSG, and 82 nM $^{13}C_4$,$^{15}N_2$-GSSG). The recovery figures of the internal standards ranged at 100% regardless which lysis protocol was applied. Accordingly, for the three investigated lysis procedures the measured GSH:GSSG ratios compared excellent with the theoretical values of the standard mixtures **[see Tab. 5]**. The experimental repeatability precision obtained by the analysis of n = 3 freshly prepared standards was 2 to 3% and 9 to 12% for the signal of the internal standards $^{13}C_2$,^{15}N-GSH and $^{13}C_4$,$^{15}N_2$-GSSG, respectively. These repeatability precisions of the procedures could be significantly improved by internal standardization. As can be observed in **[Tab. 5]**, excellent repeatability precisions of < 0.6% were observed for the determination of GSH. For GSSG 1% RSD was observed.

Sample	Intensity ratio GSH: $^{13}C_2$,^{15}N-GSH	Intensity ratio GSSG: $^{13}C_4$,$^{15}N_2$-GSSG	Intensity (GSH : $^{13}C_2$,^{15}N-GSH) / Intensity (GSSG : $^{13}C_4$,$^{15}N_2$-GSSG)	mol GSH / mol GSSG
Standard in H_3PO_4	1.4	0.3	4.4	Theoretical molar ratio: 1000
H_3PO_4cold (n = 3)	1.4	0.3	4.4	Measured molar ratio: 1023
RSD %	0.2	1.0	1.3	1.3
HCOOH cold (n = 3)	1.5	0.3	4.5	1016
RSD %	0.6	1.3	1.8	1.8
Triton X (n = 3)	1.4	0.3	4.4	1026
RSD %	0.5	1.1	0.7	0.7

Tab. 5: Intensity ratios of GSH, GSSG versus their corresponding internal standards. The adjusted concentrations of the prepared standards were 16.3 µM and 16.3 nM and 16.3 µM and 82 nM in the case of GSH and GSSG and $^{13}C_2$,^{15}N-GSH and $^{13}C_4$,$^{15}N_2$-GSSG respectively giving a theoretical molar GSH:GSSG ratio of 1000:1. A stock solution diluted in 0.1 M H_3PO_4 was employed as calibrant for the standards processed according to the 3 investigated lysis protocols.

In a next step, a comparative assessment of the lysis methods was performed by evaluation of GSH:GSSG levels in untreated HCT116 cells (n = 3 of each lysis protocol). Quantification was carried out by external calibration using the isotopically enriched internal standards. The extraction efficiency was determined as yield normalized to the highest obtained concentration level, which was achieved by cold H_3PO_4. As can be observed in **[Fig. 3 B]**, the protocol with Triton X revealed significantly lower extraction efficiency for GSSG than the acidic protocols. Notably, in some digestions using Triton X, it was not possible to recover the internal standard for GSSG (LC-MS/MS determination was <LOD), indicating that the low GSSG yields obtained in samples are not a matter of a positive assessable sample preparation protocol with low oxidation rates of GSH, than more an undesirable loss of target compound. Therefore, the Triton X-based method was excluded due to poor methodological reproducibility and robustness in the case of GSSG and $^{13}C_4$,$^{15}N_2$-GSSG.

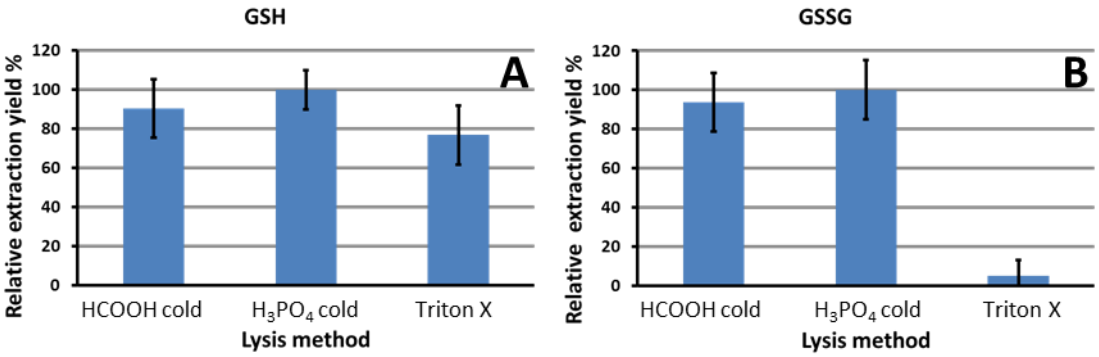

Fig. 3: Comparative assessment of 3 different lysis methods using untreated HCT116 cells as sample. The extraction yield was normalized to the highest extractable concentration level.

The findings concerning the efficiency of the other two lysis methods with respect to GSH and GSSG yield favoured neither freeze/thaw (F/T) lysis with H_3PO_4 nor HCOOH. Thus, both lysis methods were found to be equally suitable for extraction of GSH and GSSG out of cancer cell cultures for the intended purpose.

Utilization of mGSSG levels as monitoring tool for GSH oxidation

As previously mentioned the intracellular concentration of GSH is (depending on the cell line) up to 1000 times higher than the concentration of GSSG. Hence, unwanted oxidation of GSH to GSSG during cell lysis has to be controlled. For example, assuming a GSH:GSSG ratio of 100:1, already the conversion of 1% of GSH during sample processing would result in a 50% increase of the GSSG concentration levels and have a significant impact on the GSH:GSSG ratio. Thus, the implementation of a monitoring tool for the conversion of GSH to GSSG was crucial, not only for validation of the lysis methods, but for quality control during routine analysis.

In this work, the use of isotopically enriched internal standards enabled the implementation of a monitoring tool for this unwanted oxidation. More specifically, the measurement of the isotopologue denoted in the following as mGSSG, which consisted of one GSH molecule with natural isotopic distribution and one isotopically labelled GSH and could only be formed after spiking the sample, i.e. during sample preparation was exploited as a quantitative factor representing the impact of sample preparation on the determined GSSG concentration. The degree of GSH oxidation (given as %) in samples derived from HCT116 cell culture, spiked with the same concentration of $^{13}C_2$,^{15}N-GSH, was calculated by **Equation 1** using the molar concentration of the involved compounds. Preliminary experiments served for screening this concentration level. Due to the fact that the concentration level of GSH corresponded to the concentration level of $^{13}C_2$,^{15}N-GSH, **Equation 1** could be simplified to **Equation 2** (since in this case the probability of fully labelled $^{13}C_4$,$^{15}N_2$-GSSG formation corresponded to the probability of the mGSSG formation). Hence, with careful adjustment of spiking concentrations to actually present intracellular GSH levels, the oxidation degree of GSH occurring during cell lysis or just on the autosampler could be inferred by **Equation 2** and simply monitoring mGSSG.

Equation 1: $$\mathbf{(mGSSG + {}^{13}C_4,{}^{15}N_2\text{-GSSG}) / (mGSSG + {}^{13}C_2,{}^{15}N\text{-GSH} + {}^{13}C_4,{}^{15}N_2\text{-GSSG}) *100}$$

Equation 2: $$\mathbf{(2 * mGSSG)/ (2 * mGSSG + {}^{13}C_2,{}^{15}N\text{-GSH}) * 100}$$

(**mGSSG**: molar concentration of mixed oxidised glutathione, **$^{13}C_4$,$^{15}N_2$-GSSG**: molar concentration of isotopically enriched oxidised glutathione, **$^{13}C_2$,^{15}N-GSH**: molar concentration of isotopically enriched reduced glutathione)

Based on this consideration, there was no significant difference between the two acidic F/T lysis protocols with H_3PO_4 or HCOOH. As can be readily seen in **[Fig. 4]**, both sample preparation methods showed excellent figures of merit with very low oxidation of GSH. Under the applied conditions (i.e. adjusted spiking level to intracellular concentration), the mGSSG versus GSSG concentration was calculated to be 1.5% in case of HCOOH and 1.2% for H_3PO_4, respectively.

In the following preclinical investigations regarding the impact of metallodrugs on the intracellular GSH:GSSG ratio the protocol utilizing H_3PO_4 was applied. Moreover, in every following experiment the formation of mGSSG was assessed and employed as monitoring tool giving a threshold for the accurate quantification of GSH:GSSG. Exceptionally low (< 0.05%) levels of oxidation were accepted for assessment of the GSH:GSSG ratio in samples.

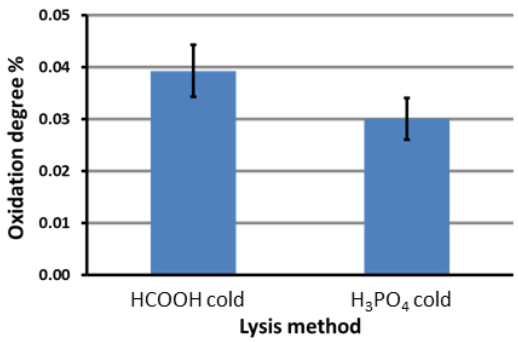

Fig. 4: Comparison of GSH oxidation degree during sample preparation and measurement (given in %, calculated according to **Equation 2** for the 2 different lysis methods (n = 3 biological replicates per lysis method). For quantification of the concentrations of mGSSG and $^{13}C_2$,^{15}N-GSH the calibrations for GSSG and GSH have been used. It was assumed that the isotopologues of GSH (monoisotopic GSH and $^{13}C_2$,^{15}N-GSH), and the isotopologues of GSSG (monoisotopic GSSG, $^{13}C_4$,$^{15}N_2$-GSSG and $^{13}C_2$,$^{15}N_1$-GSSG) respectively have the same ionisation efficiency.

Impact of metallodrugs on the intracellular GSH:GSSG ratio

It is meanwhile widely accepted that many transition metal complexes distinctly impact on the intracellular glutathione balance [56]. Here especially, the hardness/softness of the metal according to the HSAB principle seems to be a central parameter. Thus, soft acids like platinum(II), arsenic(III), or gold(I) easily react with soft bases like sulfur-containing glutathione. It is assumed that this leads to redox-independent formation of GSH conjugates and, consequently, cellular GSH pool depletion and sensibilization to reactive oxygen species (ROS). Moreover, there are several metal complexes, such as ruthenium(III) or platinum(IV), those mode of action is based on so-called "activation by reduction" processes, where intracellular reduction is supposed to result in formation of the reactive species. Glutathione as one of the most important intracellular reductants is expected to be an important players in this process. Consequently, the assessment of the intracellular GSH:GSSG ratio is a crucial tool for evaluation of the modes of action for many metallodrugs against cancer cells. In the here presented study the clinically used platinum(II) drug cisplatin as well as the experimental ruthenium(III) complex KP1339 were used. **[Tab. 6]** summarizes the obtained results for cell cultures after 24 h of drug exposure. At this point it has to be mentioned that the effect of the metallodrug in treated versus untreated control cells could only be studied when the cell culture experiments were performed within one batch. As can be readily seen two completely independent experiments on HCT116 cell resulted in different GSH:GSSG ratios already in the untreated control cells. This is not surprising as redox status of the cells is highly sensitive to many experimental parameters (such as e.g. exact cell density, age of cell culture medium, passage number). Despite this, it is safe to conclude from our data, that both investigated drugs caused a significantly reduced the GSH:GSSG ratio (increasing the reduction potential) in treated versus control cancer cells. Only in the case of KP1339 and HCT116 cells this shift was not significant. With an reported IC 50 value for KP1339 of 34.7 ± 3.6 µM, HCT116 cells showed an enhanced sensitivity to the ruthenium(III) drug compared to SW480 cells (IC 50 = 110.2 ± 31.7 µM) and other cancer cell models [69]. Untreated SW480 cells demonstrated the highest GSH:GSSG ratio, which experienced the highest change upon incubation with the ruthenium drug **[Tab. 6]**. Cisplatin showed in both investigated cell lines a comparable effect. Regarding the sensitivity towards cisplatin, IC 50 values of 1.7 ± 0.5 µM and 4.7 ± 0.1 µM were assessed for GLC4 cells and HCT116 cells, respectively [70]. In both cases an significant reduction in the GSH:GSSG ratio was encountered, suggesting that the redox state was significantly altered by incubation with cisplatin.

Cell line	Drug	mol GSH mol^{-1} GSSG	TCU %	Cell line	Drug	mol GSH mol^{-1} GSSG	TCU %
HCT116		440	10	HCT116		310	10
HCT116	cisplatin	250	15	HCT116	KP1339	280	15
GLC4		160	20	SW480		880	10
GLC4	cisplatin	90	30	SW480	KP1339	260	5

Tab. 6: Cell lines and their corresponding molar GSH:GSSG ratio after 24 h treatment with 5 µM cisplatin or 100 µM KP1339. The total combined uncertainty (TCU) represents the combined uncertainties for the measurement of GSH and GSSG and have been calculated according to the ISO GUM [71] with the postulated approach by Kragten [72] (k = 1). n = 3 biological replicates per incubation approach.

Conclusion

The experiments presented in this study clearly highlight the importance of isotopically labelled standards for assessment of accurate GSH:GSSG ratios in cancer cell models. The implementation of oxidation monitoring by measuring isotopologues mGSSG, which were generated from oxidation of internal standard was mandatory for the development of dedicated sample preparation methods. Moreover, the employed monitoring tool provided full experimental control in routine analysis in preclinical studies. Highly precise and accurate GSH:GSSG ratios could be assessed in this way, despite the fact that GSSG was present in very low concentrations. The ratios obtained in different cancer cell lines ranged from 100:1 to 800:1. The developed MS-based assay was applied to study the impact of two metallodrugs, namely cisplatin and KP1339, on the GSH:GSSG ratio using different cell models. Significant alterations of the ratio were discovered upon incubation with metallodrugs. This could be explained by disruption of the intracellular redox balance and redox stress.

Notes and references

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