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*Full Paper*

# An Investigation of Glutathione-Platinum(II) Interactions by Means of the Flow Injection Analysis Using Glassy Carbon Electrode

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**Abstract:** Despite very intensive research in the synthesising of new cytostatics, cisplatin is still one of the most commonly used anticancer drugs. Therefore, an investigation of interactions of cisplatin with different biologically important amino acids, peptides and proteins is very topical. In the present paper, we utilized flow injection analysis coupled with electrochemical detection to study and characterize the behaviour of various forms of glutathione (reduced glutathione – GSH, oxidized glutathione – GSSG and S-nitroso glutathione – GSNO). The optimized conditions were as follows: mobile phase consisted of acetate buffer (pH 3) with a flow rate of 1 mL $min^{-1}$. Based on results obtained we chose 850 mV as the optimal potential for detection of GSH and 1,100 mV as the optimal potential for detection of GSSG and GSNO. The detection limits of GSH, GSSG and GSNO were 100 pg $mL^{-1}$, 50 ng $mL^{-1}$ and 300 pg $mL^{-1}$, respectively. Further, the optimized

technique was used for investigation of interactions between cisplatin and GSH. We were able to observe the interaction between GSH and cisplatin via decrease in the signal corresponding to glutathione. Moreover, we evaluated the formation of the complex by spectrometry. The spectrometric results obtained were in good agreement with electrochemical ones.

**Keywords:** Glutathione; Carbon paste electrode; Thiols; Cisplatin; Cancer; Flow injection analysis with electrochemical detection.

## 1. Introduction

Glutathione was discovered by M. J. de Rey Pailhade at the end of the 19th century as the substance "hydrogénant le soufre", which was renamed by F.G. Hopkins in 1921. Hopkins first characterised the compound as a dipeptide of glutamic acid and cysteine. Few years later he suggested the correct structure to be a tripeptide which also contains glycine [1]. GSH as a ubiquitous tripeptide thiol is a vital intra- and extra-cellular protective antioxidant. It plays a number of key roles in the controlling of signalling processes, detoxifying of some xenobiotics and heavy metals etc. Glutathione is found almost exclusively in its reduced form; since the enzyme, which reverts it from its oxidized form (GSSG) called glutathione reductase, is constitutively active and inducible upon oxidative stress. In fact, the ratio of reduced to oxidized glutathione within cells is often used as a marker of cytotoxicity [2-5]. In connection with this, many fundamental events of cell regulation such as protein phosphorylation and binding of transcription factors to consensus sites on DNA are driven by physiological oxidant-antioxidant homeostasis, especially by thiol-disulfide balance. Therefore endogenous glutathione and thioredoxin systems and may be considered to be effective regulators of redox-sensitive gene expression [6-9].

The sulfhydryl group of glutathione is highly reactive and is often found conjugated to other molecules such as nitric oxide (NO) [10-14]. Nitrosation of the glutathione may serve as a signal event and/or as a deposition of NO to S-nitrosoglutathione. Thus, GSH can serve also as a substrate in conjugation reactions [15].

Based on the facts mentioned above it is not surprising that GSH can influence the treatment of diseases, such as cancer [16-18]. In malignant tumours, as compared with normal tissues, the multidrug resistance associates mostly with higher GSH levels within these cancer cells [19]. Thus, approaches to cancer treatment based on modulation of GSH should control possible tumour growth-associated changes in GSH content and synthesis in these cells [20-23]. An investigation of the interaction of GSH with heavy metal based cytostatics can be one of the possible ways of studying of reasons how GSH can interrupt the cancer treatment [24]. The heavy metal based cytostatics, most of all platinum complexes, play an important role in the chemotherapy of various malignancies [25-31]. The biological activity of the first platinum based cytostatic drug – cisplatin (*cis*-diamminedichloroplatinum(II)), which is still one of the most frequently used cytotoxic agents, was discovered in 1965 by Rosenborg during his studies on the effects of an electric current on bacterial growth [32]. Since then hundreds of platinum(II) and platinum(IV) complexes have been synthesized and evaluated as anticancer agents

over the past 40 years. Despite very intensive research in the synthesising of new cytostatics, cisplatin is still one of the most commonly used anticancer drugs. Therefore, an investigation of interactions of cisplatin with different biologically important amino acids, peptides and proteins is very topical. Many experimental or soft modelling methods have been proposed for these purposes,[33-39]. In the present paper, we attempted to utilize flow injection analysis coupled with electrochemical detection to study and characterize behaviour of various glutathiones (reduced – GSH, oxidized – GSSG and S-nitroso – GSNO, Fig. 1). Frther, the optimized technique was used for investigation of interactions between cisplatin and reduced GSH.

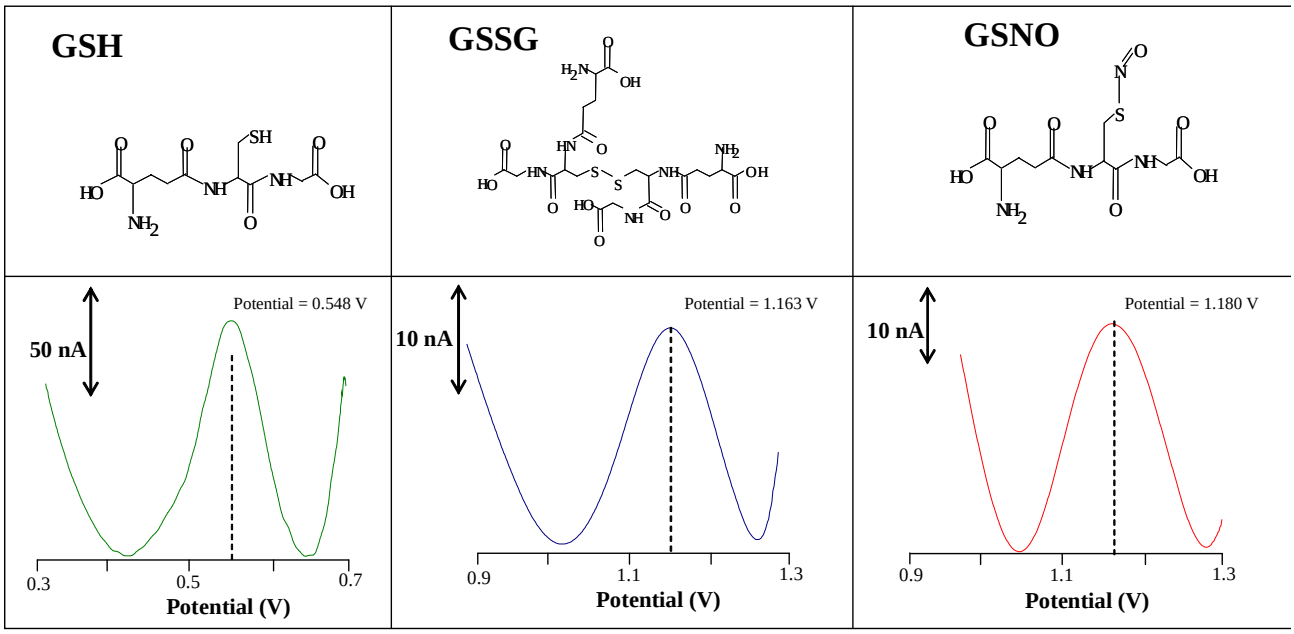


**Figure 1.** Chemical structures of reduced (GSH), oxidized (GSSG) and S-nitroso (GSNO) glutathiones. Square wave voltammograms of GSH, GSSG and GSNO measured on carbon paste electrode using AUTOLAB Analyser. The supporting electrolyte: Britton-Robinson buffer, pH 5. SWV parameters were as follows: the initial potential of –0.2 V, the end potential 1.5 V, frequency 200 Hz and step potential 5 mV. The concentration of thiols was 100 µM.

## 2. Experimental

### *2.1 Chemicals*

Reduced glutathione (GSH), oxidised glutathione (GSSG), S-nitrosoglutathione (GSNO) sodium tetraborate and other chemicals used were purchased from Sigma Aldrich (St. Louis, USA) unless noted otherwise. Tris(2-carboxyethyl)phosphine (TCEP) is produced by Molecular Probes (Evgen, Oregon, USA). Stock standard solutions of GSH, GSSG and GSNO (100 mg $mL^{-1}$) were prepared with ACS water (Sigma-Aldrich, USA) and stored in the dark at –20 °C. In addition GSH solutions were prepared in the presence of reduction agent TCEP to avoid oxidation [40]. Working standard solutions were prepared daily by dilution of the stock solutions. The pH value was measured using WTW inoLab

(Weilheim, Germany). The pH-electrode (SenTix-H, pH 0–14/3M KCl) was regularly calibrated with WTW buffers (Weilheim, Germany).

*2.2 Preparation of cisplatin solutions*

The chemotherapeutic drug of cisplatin was synthesized and provided by Pliva-Lachema (Brno, Czech Republic) [41]. Stock standard solutions of cisplatin (500 μg $mL^{-1}$) were prepared by sodium chloride solution (0.5 M, pH 6.4) and stored in the dark at -20 °C [38,42]. Working standard solutions were prepared daily by dilution of the stock solutions in phosphate buffer (100 mM).

*2.3 Electrochemical measurements*

Electrochemical measurements were performed with AUTOLAB Analyser (EcoChemie, Netherlands) connected to a VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. The working electrode was a carbon paste electrode (CPE). The reference electrode was the Ag/AgCl/3M KCl electrode and the auxiliary electrode was the graphite electrode. All experiments were carried out at room temperature. The carbon paste was made of 70 % graphite powder (Sigma Aldrich) and 30 % mineral oil (Sigma Aldrich). The carbon paste was housed in a Teflon body having a 2.5 mm diameter of active disk surface. The electrode surface was polished before each determination with a soft filter paper prior to measurement [43-45]. For smoothing and baseline correction [46], the software GPES 4.4 was employed.

*2.4 Square wave voltammetry measurements of GSH, GSSG and GSNO*

The GSH, GSSG and GSNO were measured using SWV at the surface CPE. The supporting electrolyte (Britton-Robinson buffer, pH 5) was purchased from Sigma Aldrich in ACS purity. SWV parameters were as follows: the initial potential of –0.2 V, the end potential 1.5 V, frequency 200 Hz and step potential 5 mV. For other experimental details see references [40,47].

*2.5 Preparation of GS-Pt complexes*

The GS-Pt complex was prepared according to Ishikawa and Ali-Osman [48] by incubation of GSH (60 μg $mL^{-1}$) with cisplatin (30 μg $mL^{-1}$) in 500 μl of 100 mM phosphate buffer pH 7.8 in the dark at 37 °C (thermostat BTV Brno) [49].

*2.6 Flow electrochemical measurement*

An FIA-ED system consisted of a solvent delivery pump operating in the range of 0.001-9.999 ml/min (Model 582 ESA Inc., Chelmsford, MA, USA), a guard cell (Model 5020 ESA, USA), a reaction coil (1 m) and an electrochemical detector. The amperometric electrochemical detector (ED) includes one low volume flow-through analytical cell (Model 5040, ESA, USA), which consists of a glassy carbon working electrode, a palladium electrode as a reference electrode and an auxiliary carbon electrode, and a Coulochem III as a control module. The sample (5 μl) was injected manually. The

obtained data were treated by CSW 32 software. The experiments were carried out at room temperature (22 °C). A glassy carbon electrode was polished mechanically by 0.1 μm of alumina (ESA Inc., USA) and sonicated at room temperature for 5 min using a Sonorex Digital 10 P Sonicator (Bandelin, Berlin, Germany) at 40 W.

*2.7 Spectroscopic analysis*

Spectra of GSH, cisplatin and their complexes were recorded in the presence pf 100 mM phosphate buffer (pH 7.8) using a Helios spectrophotometer (Analytic Jena, Germany).

*2.8 Statistical analysis*

STATGRAPHICS® (Statistical Graphics Corp®, USA) was used for statistical analyses. Results are expressed as mean ± S.D. unless otherwise noted. Values of $p < 0.05$ were considered significant.

## 3. Results and Discussion

*3.1 Stationary electrochemical system*

Firstly we focused on studying the basic electrochemical properties of target molecules. Reduced (GSH), oxidised (GSSG) and S-nitroso (GSNO) glutathione were measured by square wave voltammetry using carbon paste electrode. We investigated how the compounds of interest behave in the presence of Britton-Robinson buffer (pH 5) as a supporting electrolyte. We found that certain glutathiones can be determined on CPE in concentrations below 1 μM. Reduced glutathione gave oxidation signals near 0.56 V, oxidized glutathione approximately 1.16 V and nitrosoglutathione at about 1.19 V. The shift of potentials of the target molecules to more positive values can be probably related with the higher energy necessary to oxidize stronger bonds as S-S and S-NO compared to free–SH group (Fig. 1).

*3.2 Electrochemical analysis of glutathiones in flow system*

When we assessed that the glutathiones can be measured at solid electrodes (Fig. 1), flow injection analysis using glassy carbon electrode as a working one was utilized for measurement of the compounds of the interest. The flow rate of the mobile phase (Britton-Robinson, pH 5.0) was the first experimental condition we optimized. The influence of flow rate on the electrochemical responses of the target molecules was investigated within the range from 0.4 to 1.5 mL $min^{-1}$ at a working potential of 700 mV. The responses enhanced with increasing flow rate to 1.0 mL $min^{-1}$ and then slightly decrease. Based on the obtained results we chose 1.0 mL $min^{-1}$ as the optimal flow rate of the mobile phase. The observed signals were well developed and symmetrical (not shown).

3.2.1 Mobile phase composition and its pH

Other experimental conditions such as composition of mobile phase and its pH were also optimized. We choose three various buffers (acetate, phosphate and Britton-Robinson) and investiagted

how their different pH values can affect the electrochemical responses of glutathiones (Fig. 2). It clearly follows from the results obtained that acetate buffer (pH 3) was the most suitable mobile phase to measure glutathiones with the highest sensitivity. If phosphate buffer was used, the lowest signals were measured. Particularly, the peak heights of glutathiones measured in the presence of phosphate buffer were about 80 % lower than in acetate buffer. Similarly, if we used Britton Robinson buffer as the mobile phase signals were about 20-60 % lower than in acetate buffer. It clearly follows from the obtained results that the electrochemical responses of glutathiones measured on a glassy carbon electrode are affected not only by the composition of the mobile phase but also by its pH (Fig. 2). Moreover it can be concluded that the higher pH values used, the lower the electrochemical responses of compounds of interest were observed.

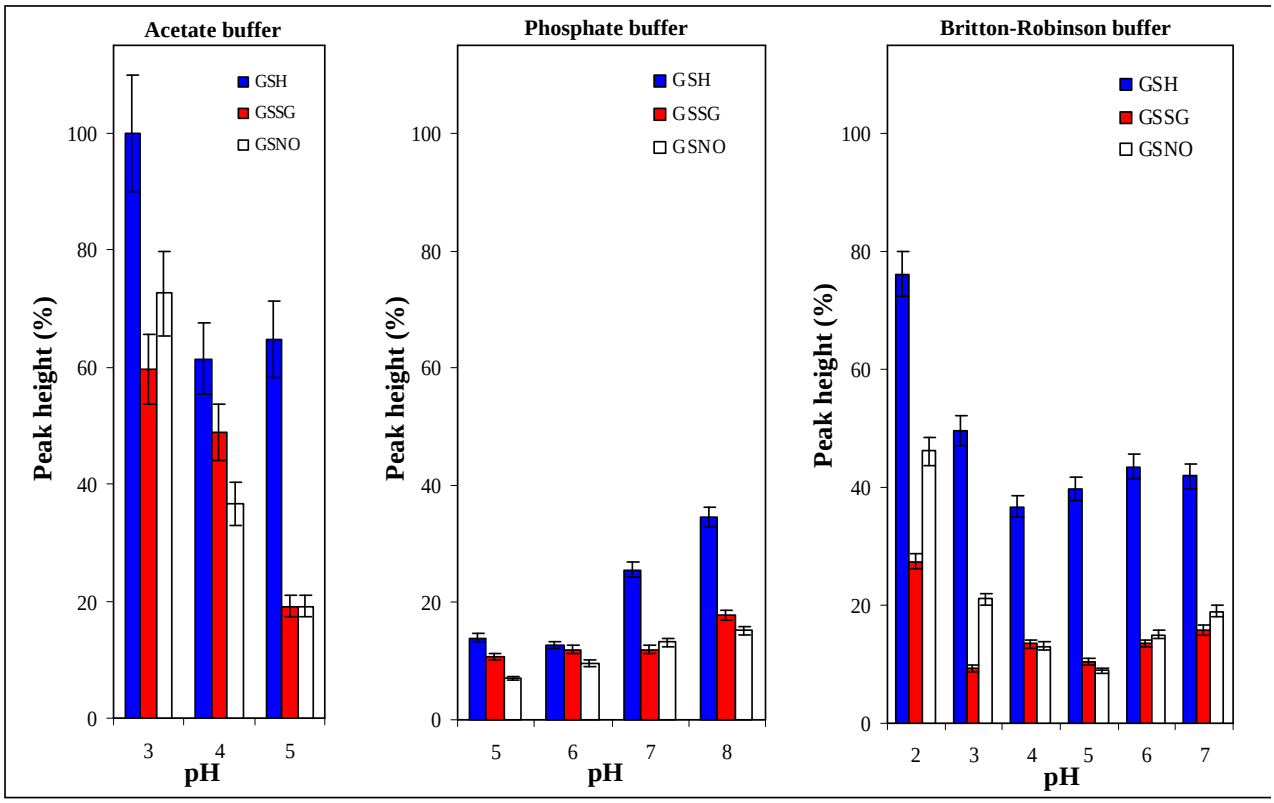


**Figure 2.** Influence of pH of acetate, phosphate and Britton-Robinson buffers on electrochemical responses of GSH, GSSG and GSNO. Concentration of the buffers: 0.2 M. Flow rate of electrolyte was 1.0 ml.min$^{-1}$, potential 700 mV. Signal of GSH measured in the presence of acetate buffer (pH 3.0) of height 468 µA corresponds to 100 %. The concentration of thiols was 30 µg mL$^{-1}$.

3.2.2 Applied potential on glassy carbon working electrode

As we have optimized flow rate of a mobile phase and its composition, a working electrode potential was the last experimental conditions we optimized. We measured glutathiones of the same concentration (30 µg mL$^{-1}$) in the presence of acetate buffer (pH 3) with a flow rate of 1 mL min$^{-1}$ at various working electrode potentials. The hydrodynamic voltammograms obtained are shown in Fig. 3. If GSH was measured, the rapid increase of the signal with maximum at 900 mV was observed. The

increase of the electrochemical responses of GSSG and GSNO were observed at potentials higher than 800 mV. The peaks increased to 1,300 mV, where the highest peaks of GSSG and GSNO were observed. If we compared the potentials of the highest peaks of glutathiones measured by flow technique to those measured by SWV, we found that the potentials measured by flow technique shifted slightly to more positive values (Fig. 1 and Fig. 3). The shift can be associated with different reference and working electrodes used (hydrogen-palladium in flow system and Ag/AgCl, 3M KCl in stationary). Based on the results obtained we choose 850 mV as the optimal potential for detection of GSH and 1,100 mV as the optimal potential for detection of GSSG and GSNO. The signals of GSSG and GSNO measured under potentials higher than 1,100 mV were higher but the stabilizing of background currents lasted more than twenty minutes and standard deviations exceeded 10 %. These disadvantages encouraged us to utilize 1,100 mV for detection of GSSG and GSNO in the following experiments.

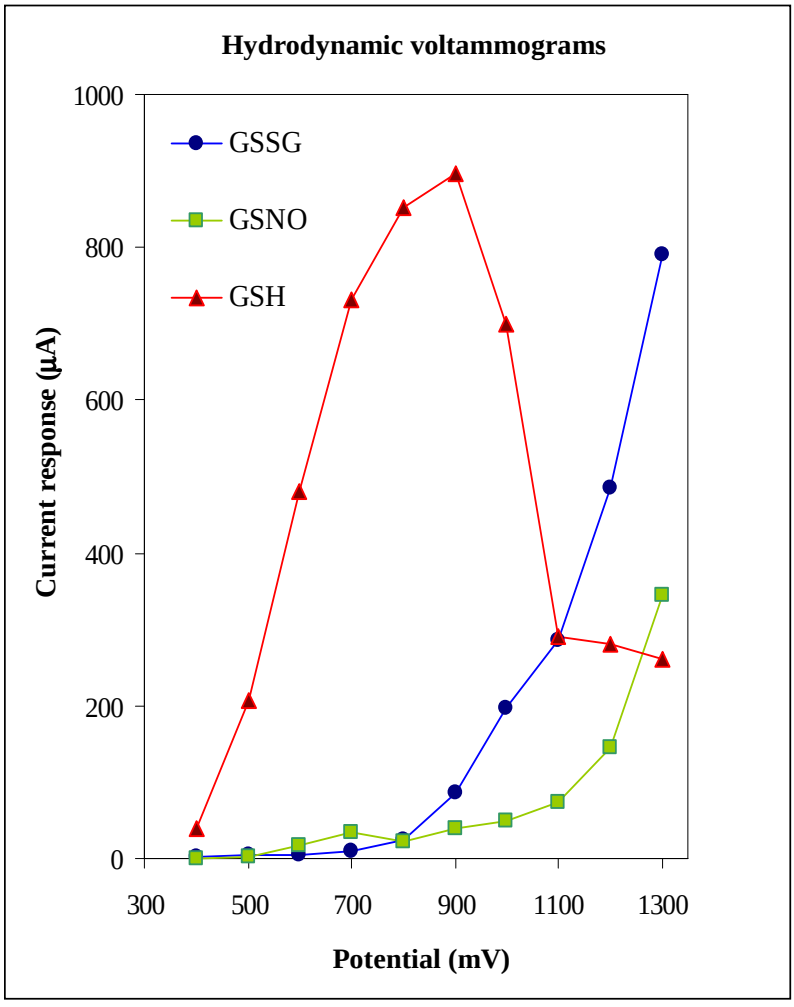


**Figure 3.** Hydrodynamic voltammograms of GSH, GSSG and GSNO measured on glassy carbon electrode. Mobile phase: 0.2 M acetate buffer. For other details see Fig. 2.

3.2.3 Calibration curves

Under the experimental conditions optimized above we investigated the influence of glutathiones concentration on their current responses. The dependences measured are shown in Figs. 4A,B,C. The

dependences were nonlinear within the concentration ranges we studied, whereas the linear ranges of the dependences are shown in insets in Figs. 4A,B,C. The nonlinearity observed can be associated with an accumulation of analyte on the electrode surface, where poly-layers of analyte adsorbed could form [50]. In addition, the signals were very well reproducible with a relative standard deviation of 2-5%.

GSSG gave the lowest current responses in comparison with other compounds of interest. The concentration dependence of GSSG was linear (y = 5.4816x + 0.4914; $R^2$ = 0.9939) within the concentration range from 0.1 to 2.5 µg $mL^{-1}$, with a detection limit of 50 ng $mL^{-1}$. If we measured GSNO, we obtained strictly linear concentration dependence (y = 0.0052x + 0.2099; $R^2$ = 0.9957) within the range from 1 to 500 ng $mL^{-1}$, whereas the detection limit was 300 pg $mL^{-1}$. In addition, GSH concentration dependence was strictly linear (y = 11.315x - 0.5146; $R^2$ = 0.9969) within the range from 0.5 to 4 ng $mL^{-1}$. The detection limit of GSH was 100 pg $mL^{-1}$ (Fig. 4). The detection limits were evaluated as 3 S/N ratio according to Long and Winefordner [51].

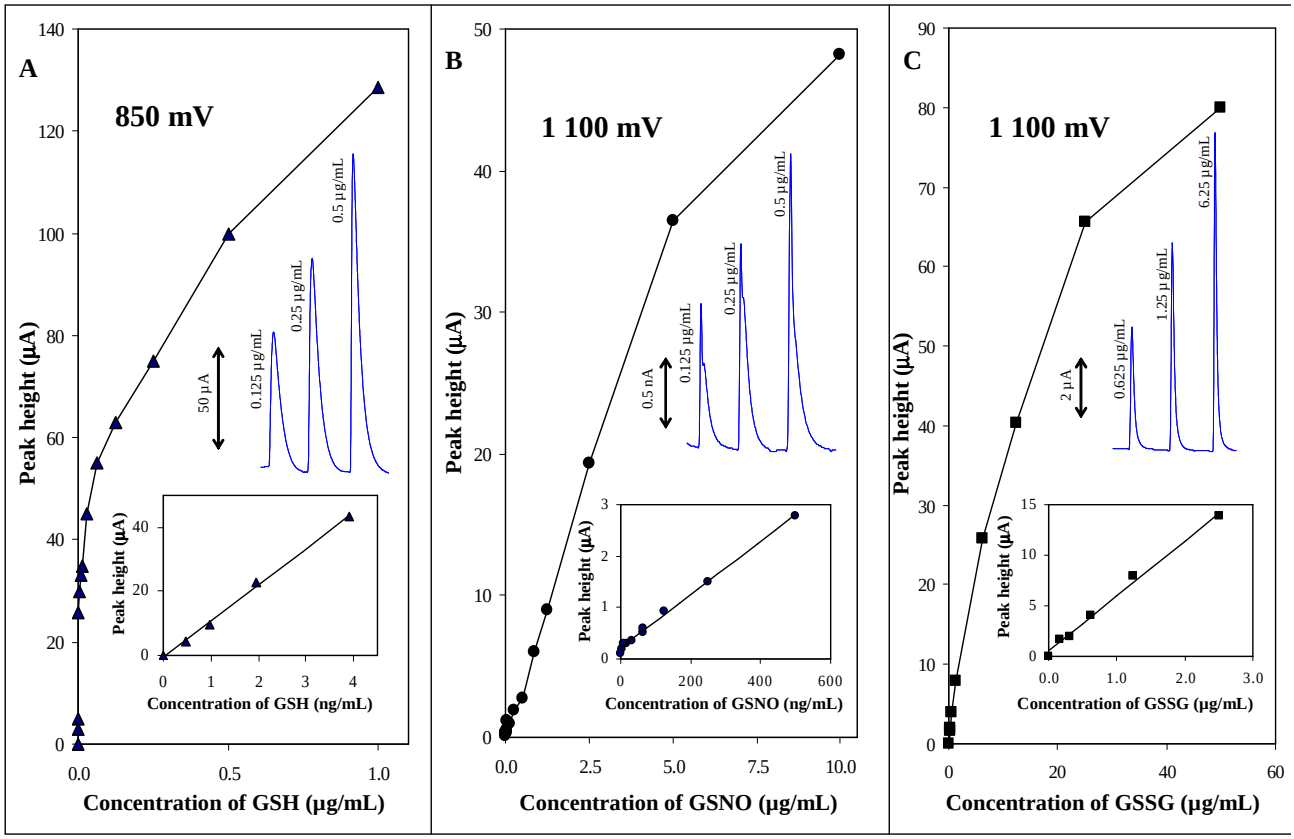


**Figure 4.** Dependences of peak heights of GSH (**A**), GSSG (**B**) and GSNO (**C**) on their concentrations; insets: calibration dependences of the thiols and real chromatograms. For other details see Fig. 3.

### *3.3 Study of GSH-cisplatin interaction*

GSH belongs to group of thiols, like metalothioneins, which could most likely influence therapeutic concentration of platinum based cytostatics (e.g. cisplatin) within a body and, therefore, could play one of the key roles in formation of a resistance on the treatment [52-57]. A mechanism of action of GSH with cisplatin is still unclear, but the most accepted opinion is that the thiol decreases of therapeutic concentration in the place of the drug action [58-60]. The contribution of other forms of glutathiones

including GSNO and/or GSSG to decrease of therapeutic concentration of platinum based cytostatics is thought to be negligible due to their few folds lower concentration and reactivity. Thus, studying of interactions between these low abundant glutathiones with cytostatics is of low clinical importance. Nevertheless, investigating of interactions between GSH and cytostatics if of high importance but it is very difficult and needs robust and high cost instruments as nuclear magnetic resonance.

Here, we propose a simple method to characterize the interactions of GSH with cisplatin. Therapeutic doses of cisplatin (3, 15 and 30 µg $mL^{-1}$) were used for this purpose. GS-Pt complexes were prepared according to Ishikawa and Ali-Osman [48]. We observed the interaction between ciplatin and GSH by determining of changes in GSH signal. As expected, the formed GS-Pt complex gave lower responses compared to reduced glutathione (Fig. 5A). Therefore, the hydrodynamic voltammograms of GSH and GS-Pt complex measured in the range from 400 to 1,100 mV differed. The current response of GS-Pt complex increased slowly up to an applied potential of 750 mV, then the rapid increase of the signal with maximum at 900-950 mV occurred (Fig. 5A). Therefore we further measured the GS-Pt complex at 950 mV. Moreover, the signal of cisplatin (30 µg $mL^{-1}$) under these experimental conditions was negligible.

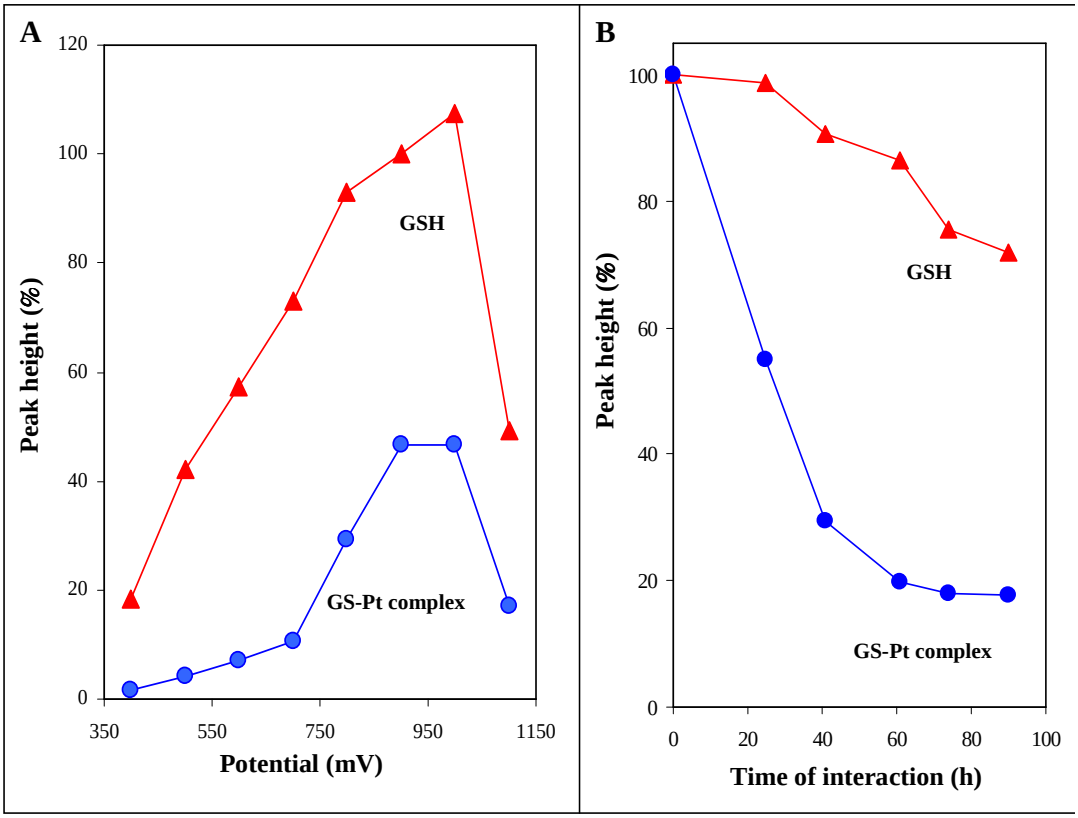


**Figure 5.** Hydrodynamic voltammograms of GSH and GS-Pt complex (1 µg $mL^{-1}$) after 60 h long interactions (**A**). Dependences of height of GSH and GS-Pt complex on time and time of interaction, respectively (**B**). Signal of GSH of height 46.7 µA (**A**) and of height 1,620 µA (**B**) corresponds to 100 %. Cisplatin (30 µg $mL^{-1}$) and GSH (60 µg $mL^{-1}$) interacted in the presence of phosphate buffer (pH 7.8) in the dark at 37 °C. For other details see Fig. 4.

During time scale investigating of formation complexes between GSH and cisplatin we also studied if the height of GSH peaks without cisplatin can change. We found the peak of GSH decreased gradually within the range of time we were interested in (Fig. 5B). In addition, the dependence of peak height of GS-Pt complex on times of interactions is shown in Fig. 5B. It clearly follows from the results obtained that dependences differed markedly, which confirmed that we really observed the interactions between cisplatin and GSH. The signal of complex decreased more than 40 % during the 20 h long interaction at 37 °C. Moreover, after a 60 h long interaction the maximum yield of complex had been reached. We also decided to evaluate the formation of complex by another reference technique. The complex formation was monitored spectrometrically as absorbance change at 280 nm [49]. The spectrometric results obtained were in good agreement with electrochemical ones.

## Conclusion

Electrochemical detection represents an alternative method for determination of biologically active compounds [42,61-71]. Here, we have shown the flow injection analysis coupled with electrochemical detector could be a suitable technique not only for direct detection of various forms of glutathione but also for investigating of interactions between thiols and cytostatics. FIA-ED is very promising tool for such purposes because it is low cost and very sensitive to various electroactive species.

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