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Optimization of the Glutathione Detection by High Performance Liquid Chromatography with Electrochemical Detection in the Brain and Liver of Rats Fed with Taurine

Marketa Kominkova[1,2], Pavel Horky[3], Natalia Cernei[1,2], Katerina Tmejova[1,2], Branislav Ruttkay-Nedecky[1,2], Roman Guran[1,2], Miroslav Pohanka[2], Ondrej Zitka[1,2], Vojtech Adam[1,2], Rene Kizek[1,2,]*

[1] Department of Chemistry and Biochemistry, Laboratory of Metallomics and Nanotechnology , Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic, European Union
[2] Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, CZ-616 00 Brno, Czech Republic, European Union
[3] Department of Animal Nutrition and Forage Production, Faculty of Agronomy, Mendel University in Brno, Brno, Czech Republic
[*]E-mail: kizek@sci.muni.cz

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Taurine exhibits many positive effects on organism, such as induction of important protective biochemical mechanisms and antioxidant effects. In this work, we studied the effect of taurine consumption on levels of taurine, reduced glutathione (GSH) and metallothionein in liver and brain tissues of Wistar rats. The aim of the experiment was the monitoring of changes in concentration of taurine in brain and liver tissues and also changes in concentration of GSH and metallothionein. After optimization, the limit of detection of GSH in brain and liver tissue was established as 40 nmol.g^{-1} of protein and 90 nmol.g^{-1} of protein, respectively. That is sufficient for determination of the concentration alterations of GSH in studied tissues. The consumption of taurine increased content of GSH determined in the brain and liver (increased by approximately 0.8 mmol.g^{-1} of protein in comparison with control). The increase of concentration of taurine occurred only in the brain, by cca 50 μg.g^{-1} of tissue, regardless the applied concentration of taurine. The applied concentration of taurine had no effect on concentration of metallothionein in any of the examined tissue.

Keywords: glutathione, taurine, metallothionein, antioxidant, amino acids, rats, HPLC

1. INTRODUCTION

Although taurine (2-aminoethanesulfonic acid) is often referred to as amino acid, it is the only known naturally occurring sulfonic acid [1,2]. In human body it is biosynthesized in liver from

methionine or cysteine by their decarboxylation and subsequent oxidation of the sulfhydryl group [2,3]. At lower concentrations it can be found practically in all animal tissues. In bile acids it acts as a surface tension reducing agent. Taurine has many fundamental biological roles, such as, antioxidation, osmoregulation, membrane stabilization, and modulation of calcium signaling [4].

Higher levels of taurine are also located in the brain and in skeletal muscle cells and heart [5-7]. Taurine also exhibits antioxidant activity and has the ability to lower blood malonyldialdehyde levels in the organism [8]. The most intense elevation of both metabolic (lactate dehydrogenase, malate dehydrogenase) and antioxidant activity (catalase) after feeding of taurine-enriched diet were detected in the liver and in the anterior intestine of the fish *Dicentrarchus labrax* showing that taurine induces important protective biochemical mechanisms [9]. In another study, Anand et al. [10] investigated the effect of oral administration of taurine on activity of antioxidant enzymes in rats. Glutathione peroxidase activity showed an increase in liver, heart, stomach and plasma. Glutathione reductase activity increased in kidney and decreased in liver and plasma. Glutathione (GSH, (2S)-2-amino-4-{[(1R)-1-[(carboxymethyl)carbamoyl]-2-sulfanylethyl]carbamoyl}butanoic acid) levels increased in liver, stomach and decreased in kidney. Taurine improves the utilization of protein and fat, increasing energy use of these nutrients. It also contributes to the reduction of low-density lipoprotein cholesterol in the blood [11]. Taurine administration increases the production of alkaline phosphatase, which binds to osteopontin and increases bone strength. Taurine also has a strong neuroprotective function through stimulation of neuronal receptors in the brain [12]. Method for GSH determination using electrochemical detection, either polarographic and voltammetric techniques have already been published [13,14]. Electrochemical detection is often used in particularly in connection with separation techniques. This link is also suitable for the detection of other glutathione derivatives. For electrochemical detection of glutathione it is appropriate to use higher potential >700 mV [15-17].

The aim of this study was to optimize the flow injection analysis/ high performance liquid chromatography – electrochemical detection (FIA/HPLC-ED) method for determination of GSH and to apply it for the analysis of rat brain and liver samples. GSH is a substrate of many antioxidant enzymes and itself is also a strong antioxidant. Therefore, its connection in antioxidation system with taurine, which is characterized by similar functions in organism, is close. These analytes were chosen in order to assess the impact of taurine, which was used as a feed, on the antioxidant status of organs. The antioxidative effect of taurine was assessed by determining terminal antioxidative indicators: concentration of oxidized and reduced GSH and concentration of metallothionein.

2. EXPERIMENTAL PART

2.1. Chemicals and pH measurement

Glutathione, taurine and other chemicals were purchased from Sigma Aldrich. (St. Louis, Missouri, USA), unless noted otherwise. The deionised water was prepared using reverse osmosis equipment Aqual 25 (Czech Republic). The deionised water was further purified by using apparatus

MiliQ Direct QUV equipped with the UV lamp. The resistance was 18 MV. The pH was measured using pH meter WTW inoLab (Weilheim, Germany). Methyl cellosolve and tin chloride were purchased from Ingos (Prague, Czech Republic).

2.2. Laboratory animals and their keeping

In total, 60 males of Wistar rats (Biotest, Konarovice, Czech Republic) were used in the experiment. At the beginning of experiment, the animals were 28 days old and differences in body weight were in a range of 2.5 g. The animals were kept in an air conditioned room with stable temperature of 23 ± 1°C, humidity 60%, light period 12 h with light intensity up to 200 lx, content of CO_2 up to 0.25%, NH_3 up to 0.0025%. Food and water were provided *ad libitum* during the whole experiment. All animals were in good condition and no aberrations in behaviour and development were observed. The experiment was approved and supervised by ethical committee, Mendel University in Brno, Brno, Czech Republic.

2.3. Experimental design

Animals were divided into 8 experimental groups (5 males in each group). They were supplemented with taurine through feed mixture, which was enriched by taurine in the dosage of 0, 35, 52.5, 70, 78.5, 105, 122.5 and 140 $mg.day^{-1}$. Animals were euthanized after 28 days. Then samples of brain and liver were collected, frozen and subsequently analysed.

2.4. Sample preparation for determination of total protein, taurine, glutathione and metallothionein

A sample (1 g of liver or brain, fresh weight) was deeply frozen by liquid nitrogen. After that, 1 mL of 0.2 M phosphate buffer (pH 7.0) was added. Sample was vortexed for 15 min and centrifuged at 25000 g (20 min at 4°C). A volume of 300 µL of supernatant was taken and mixed with 300 µL of 10% trifluoroacetic acid (TFA). Subsequently the sample centrifuged (24000g, 20 min, 4°C). Supernatant was used for analyses. Samples were stored on the ice all the time.

2.5. Determination of total protein

Determination of total protein content using Bradford method is described in the following paper [18]. Briefly, reagent Coomassie Brilliant blue G-250 (0.01% Coomassie Brilliant Blue G-250, 4.7% CH_3CH_2OH, 8.5% H_3PO_4, v/v) in volume of 190 mL was pipetted into cuvette. Further, sample (10 mL) was added. Mixture was incubated at 37°C for 10 min. Absorbance was measured at 595 nm and reagent itself was used as a blank.

2.6. Determination of reduced glutathione

FIA/HPLC-ED system consisted of two chromatographic pumps Model 582 ESA (ESA Inc., Chelmsford, MA) (working range 0.001-9.999 $mL.min^{-1}$) and chromatographic column with reverse

phase Zorbax eclipse AAA C18 (150 × 4.6 mm; 3.5 μm particles, Agilent Technologies, USA) and twelve-channel CoulArray electrochemical detector (Model 5600A, ESA, USA). Detector consisted of three flow analytical chambers (Model 6210, ESA, USA). Each chamber contained four analytical cells. One analytical cell contained two referent (hydrogen-palladium), two counter and one porous graphite working electrodes. Electrochemical detector was situated in control module which was thermostated. Sample (20 μL) was injected by autosampler (Model 542, ESA, USA), which had thermostated space for column [19]. Conditions for HPLC-ED analysis were: Column was termostated at 35°C. Flow rate of mobile phase was 1 $mL.min^{-1}$. Mobile phase consisted of A: trifluoroacetic acid (80 mM) and B: 100% Met-OH. Compounds were eluted by following linear increasing gradient: 0→1 min (3% B), 1→2 min (10% B), 2→5 min (30% B), 5→6 min (98% B). Detection was carried out at applied potential 900 mV. Time of one analysis was 20 minutes [8,20-28].

2.7. Taurine determination

For determination of taurine, an ion-exchange liquid chromatography (Model AAA 400, Ingos, Czech Republic) with post-column derivatization with ninhydrin and VIS detector was used. A glass column with inner diameter 3.7 mm, and 350 mm in length was filled manually with a strong cation exchanger in sodium cycle LG ANB (Ingos) with approximately 12 μm particles and 8% porosity. The glass column was tempered within the range from 35 to 95°C. A double-channel VIS detector with the volume of flow cuvette of 5 μL was set to two wavelengths – λ = 440 and 570 nm. A solution of ninhydrin (Ingos) was prepared in the mixture of 75% (*v/v*) methylcelosolve (Ingos) and 25% (*v/v*) 4 M acetate buffer (pH 5.5). Stannous chloride ($SnCl_2$, Lachema, Czech Republic) was used as a reducing agent. The prepared solution of ninhydrin was stored under an inert atmosphere (N_2) and cooled at 4 °C. Elution of amino acids was performed according to program using a discontinuous gradient of elution buffers of different ionic strength and pH, and also using a temperature gradient. During the analysis, the flow rate was 0.3 $mL.min^{-1}$ under the pressure of 4.5-6 MPa. Temperature was set to 120°C in the heat generator. Temperature was set to 60°C in the column [29].

2.8. Determination of metallothionein

Differential pulse voltammetric measurements were performed with 747 VA Stand instrument connected to 693 VA Processor and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and cooled sample holder and measurement cell to 4ºC (Julabo F25, JulaboDE, Germany). A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3 M KCl electrode was the reference and platinum electrode was auxiliary one. For data processing VA Database 2.2 by Metrohm CH was employed. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999%) saturated with water for 120 s. Brdicka supporting electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer ($NH_3(aq)$ and NH_4Cl, pH = 9.6) was used. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement were: initial potential of -0.700 V, end potential of -1.750 V, modulation time 0.057 s, time interval 0.2 s, step potential 0.002 V, modulation amplitude -0.250 V,

Eads = 0 V, volume of injected sample 20 µL, volume of measurement cell 2 mL (20 µL of sample and 1980 µL Brdicka solution) for calibration curves [30].

2.9. Descriptive statistics

Mathematical analysis of the data and their graphical interpretation were realized by Microsoft Excel®, Microsoft Word® and Microsoft PowerPoint®. Results are expressed as an average ± standard deviation (S.D.) unless noted otherwise. The detection limits (3 signal/noise, S/N) were calculated according to Long and Winefordner [31], whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

3. RESULTS AND DISCUSSION

Amperometric detection was earlier used for analysis of thiol compounds in lettuce [32]. We had to optimize FIA-ED method for evaluation the influence of preparation of animal tissue samples and of substances contained in these tissues on the amperometric detection and eventual GSH oxidation. The arrangement of FIA-ED is shown in Fig. 1. Trifluoroacetic acid (80 mM) was propelled into the FIA system by chromatographic pumps (Fig. 1A) at a flow rate 1 mL.min^{-1}. A sample of GSH or tissue supernatant was injected (Fig. 1B) into the continuous flow of carrier phase and subsequently a response of electrochemical detector at applied potential was evaluated (Fig. 1C, D).

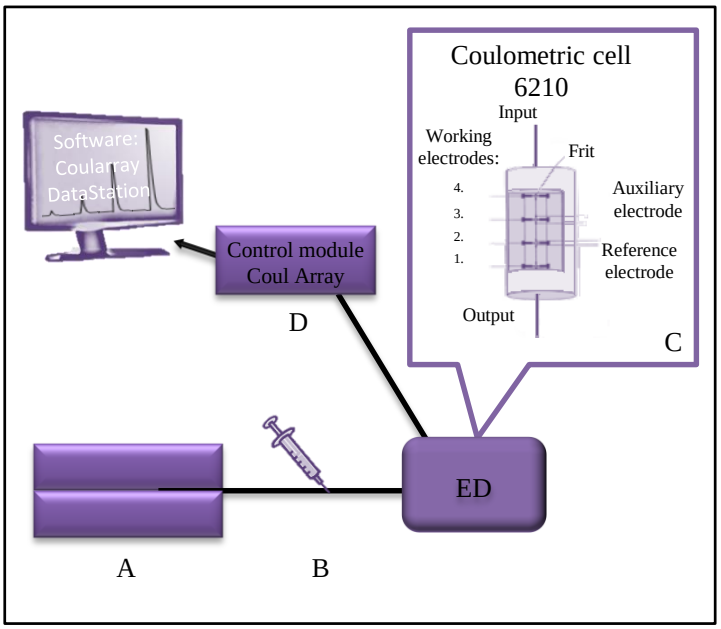

Figure 1. Scheme of FIA-ED. The carrier phase (80 mM TFA) is propelled by chromatographic pumps (**A**). A sample is injected (**B**) into continuous flow of carrier phase and then is carried straight to analytical cells (**C**). The response of analytical cell is transferred into PC by control module (**D**).

Firstly, hydrodynamic voltammograms (HDVs) of GSH (100 µg.mL^{-1}), tissues (liver and brain) and tissues with addition of GSH (100 µg.mL^{-1}) were evaluated (Fig. 2A, B). These results show that for amperometric detection of GSH is optimal 900 mV potential because at this point the highest peak height was achieved. This finding corresponds to previously published paper, which also reported a 900 mV potential as ideal for detection of GSH [32]. Besides that, HDVs of liver and brain tissue samples had maximal peak heights at applied potential 1100 mV. This difference in the optimal applied potential is also advantageous for the subsequent chromatographic analysis, when during the separation of the peaks is achieved better resolution than it would be if the optimal potentials of GSH and tissue samples were same. The sample prepared from liver contained 1.2 g.L^{-1} of total proteins and the sample prepared from brain contained two times lower concentration of total proteins, 0.6 g.L^{-1}. The addition of GSH caused no change in a concentration of total proteins.

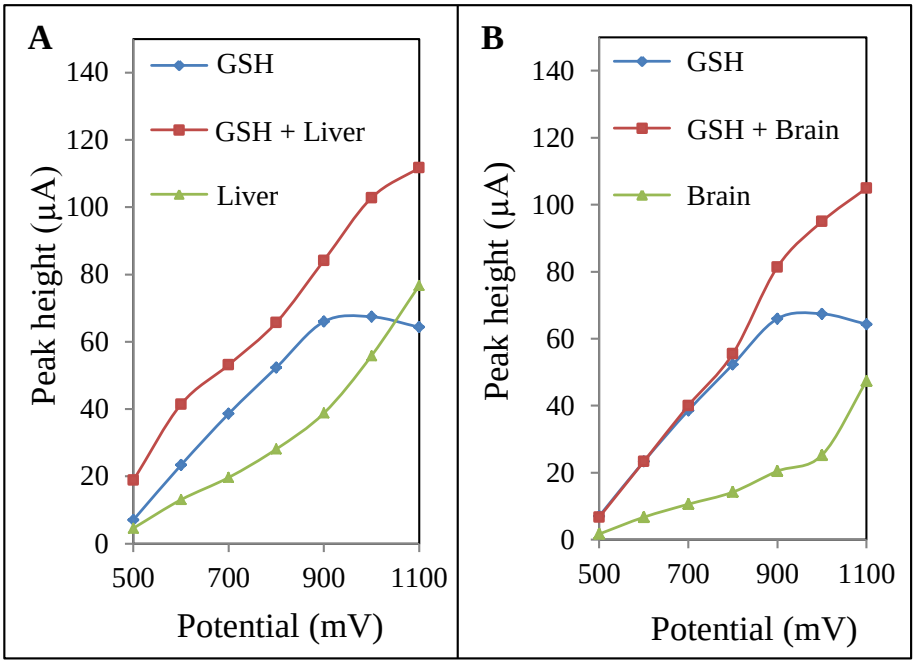

Figure 2. HDVs from optimization of GSH detection using FIA-ED at flow rate 1 mL.min^{-1}. The influence of applied potential on peak height of GSH (100 µg.mL^{-1}), tissues and tissues with addition of GSH (100 µg.mL^{-1}). (**A**) Sample of liver tissue. (**B**) Sample of brain tissue. HDVs were created in a range 500-1100 mV with a potential step of 100 mV. The volume of injection loop was 20 µL. Each point in graphs represents an average from three measurements.

The calibration solutions of GSH were prepared alone and also in tissue supernatants. A 900 mV potential was used for amperometric detection. Samples for HPLC-ED were prepared in a same way as was described in previous step, but different concentrations of GSH in a range 0.8-100 µg.mL^{-1} were added to tissue supernatants (Fig. 3A, B). The high value of intercept in case of tissue samples is caused by naturally occurring GSH in tissues.

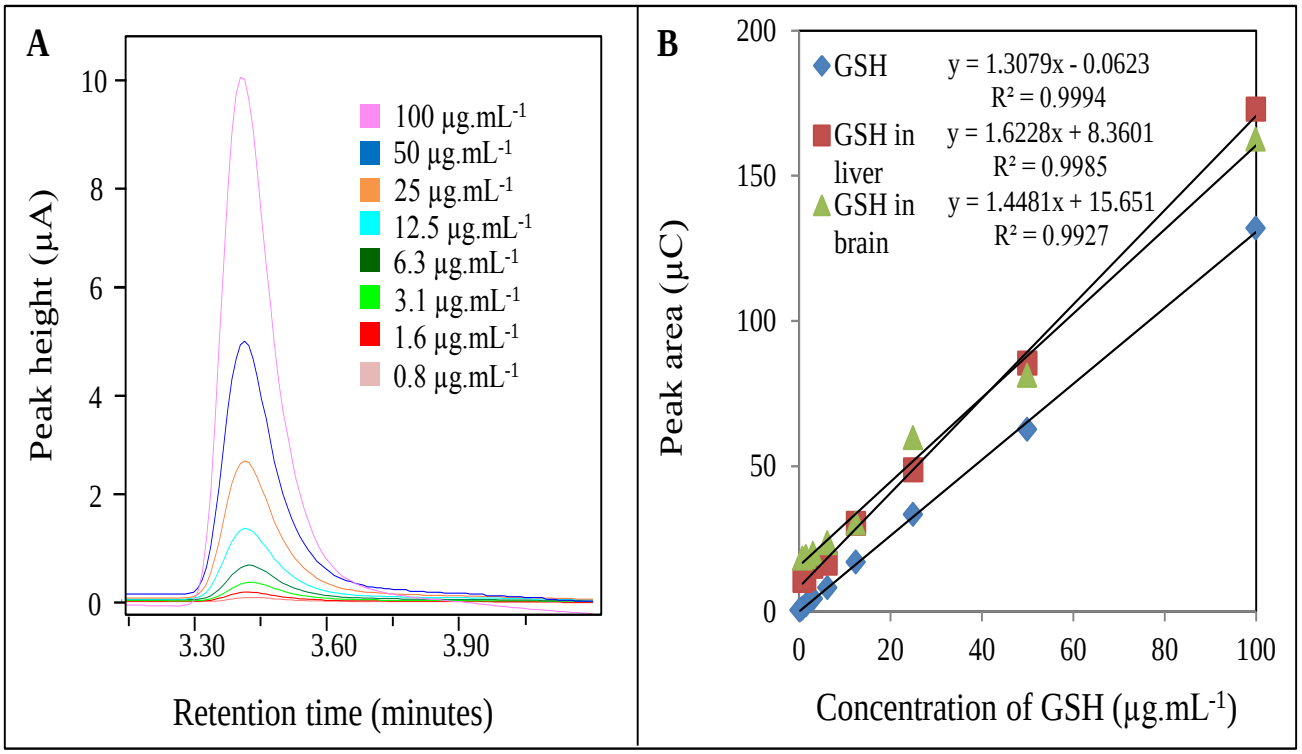

Figure 3. (**A**) Chromatogram of GSH calibration standards (0.8; 1.6; 3.1; 6.3; 12.5; 25; 50 and 100 µg.mL^{-1}) from HPLC-ED at 900 mV. (**B**) Dependence of signal on GSH concentration for GSH (blue diamond), GSH in liver (red square) and GSH in brain (green triangle)

Limit of detection (LOD, 3 S/N) was calculated from calibration curves and its value for GSH is 60 nM, for GSH in liver supernatant is 50 nM and for GSH in brain supernatant is 50 nM. Next, analytical parameters as regression equation, linear dynamic range, LOD and limit of quantification (LOQ) are shown in Tab. 1. LODs of GSH standard and GSH in the tissue environment is virtually unchanged, the same applies to other specified analytical parameters. Sample preparation and detection method does not affect the detection of GSH in tissues, even in low concentrations. We also carried out repeatability and intermediate measurement precision data estimation (Tab. 2). Intermediate measurement precision was evaluated by determining five replicates during three consecutive days (n = 15). The results showed good average of relative recoveries within the range from 97 to 99% with good RSD of less than 3.9% in repeatability analysis and less than 4.8% for intermediate measurement precision. These results show that this method is suitable for analysis of tissue as the concentration of GSH is naturally about 2 mM in a brain and 7 mM in a liver. Similar range of GSH concentrations were observed also in other tissues [33,34]. From the calibration curves of taurine and metallothionein (Fig 4 A, B) follows the LOD for taurine 1 µM and for metallothionein 0.1 µM, other analytical parameters such as linear dynamic range and LOQ are summarized in Tab. 3.

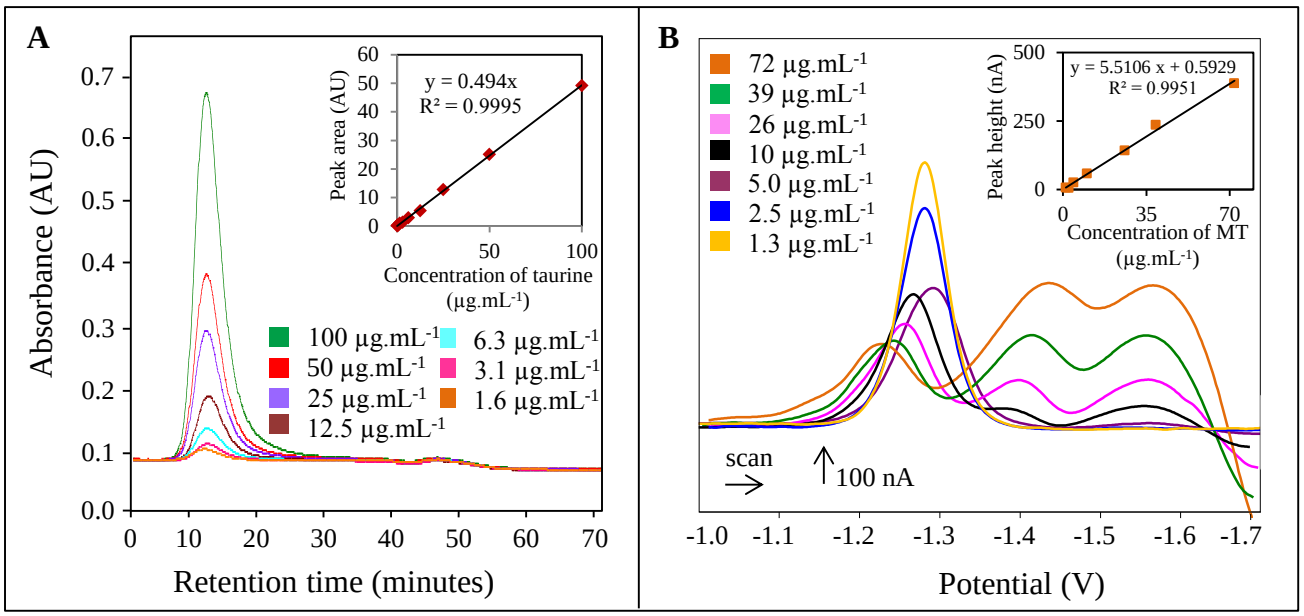

Figure 4. (**A**) Chromatogram of taurine calibration standards (1.6; 3.1; 6.3; 12.5; 25; 50, and 100 µg.mL^{-1}) from ion-exchange liquid chromatography with post-column derivatization by ninhydrin. (**B**) Voltammograms of metallothionein calibration standards (1.3, 2.5, 5, 10, 26, 39 and 72 µg.mL^{-1}) from differential pulse voltammetry. Insets show calibration curves of analytes.

Table 1. Analytical parameters of HPLC-ED detection of GSH (100 µg.mL^{-1}) in tissue: retention time, regression equation, linear dynamic range, coefficient of determination (R^2), LOD (3 S/N), LOQ (10 S/N); injection volume - 20 µL.

Compound	Retention time	Linear regression Equation	Linear dynamic range	R^2	LOD	LOD per injection	LOQ	LOQ per injection
	(min)		(µM)		(nM)	(pmol)	(nM)	(pmol)
GSH	3.9	y = 1.3082x - 0.0822	2-325	0.9993	60	0.3	200	0.1
GSH in liver	3.9	y = 1.6228x + 8.3601	1.6-325	0.9985	50	0.2	160	0.8
GSH in brain	3.9	y = 1.4481x + 15.651	1.8-325	0.9927	50	0.3	180	0.9

Table 2. Repeatability and intermediate measurement precision and accuracy of HPLC-ED, where RSD stands for relative standard deviation.

Compound	Concentration	Repeatability (*n*=8)	Recovery	Intermediate measurement precision (*n*=15)	Recovery
GSH	(µg.mL^{-1})	RSD (%)	(%)	RSD (%)	(%)
	25	3.85	98	4.81	99

	50	3.72	99	3.69	97
	100	3.21	97	3.17	97

Table 3. Analytical parameters of taurine and metallothionein: coefficient of determination, LOD (3 S/N), LOQ (10 S/N).

Compound	Linear regression Equation	Linear dynamic range	R^2	LOD	LOQ
		(µM)		(µM)	(µM)
Taurine*	y = 0.494x	4-800	0.9995	1	4
Metallothionein**	y = 5.5106x + 0.5929	0.2-12	0.9951	0.1	0.3

***** Taurine levels were determined by using ion-exchange chromatography with post-column derivatization with ninhydrin and VIS detection, **Metallothionein levels were assessed by using differential pulse voltammetry.

The method described above was applied for monitoring the effect of taurine, included in the feed ration for rats, on level of antioxidant compounds in the liver (the key organ of metabolism) and in the brain (organ with the biggest concentration of taurine in the body). In the experiment, 8 rat groups, which were fed with taurine in the concentration range from 0 to 140 mg of taurine per day for 28 days, were investigated. Subsequently, the rats were euthanized and the organs were analysed. Not only GSH, but also concentrations of taurine and metallothionein in the organs were monitored. These molecules were observed due to significant antioxidant activity of taurine and they were used as indicators of antioxidant status of the organs [8,35,36].

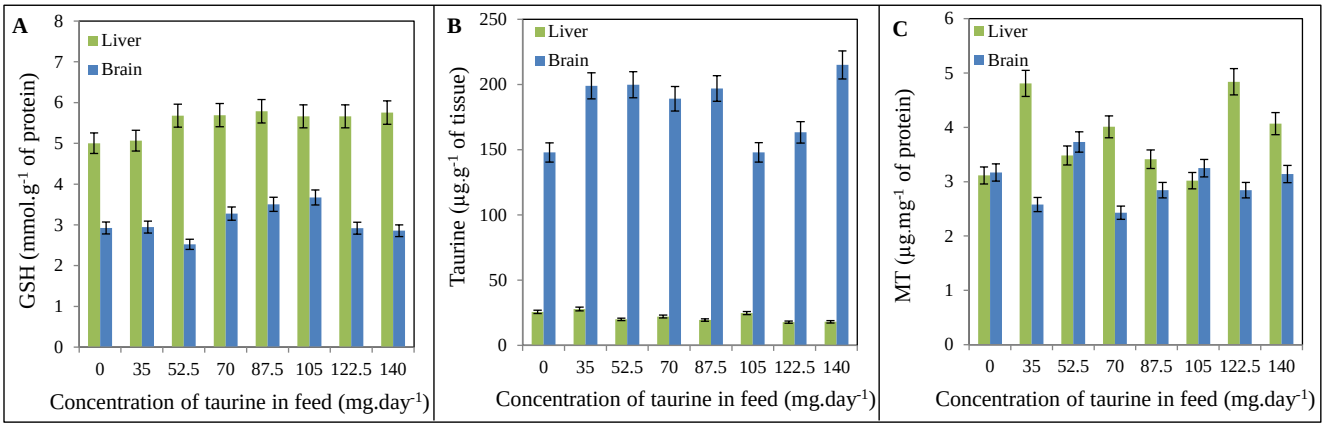

Figure 5. Graphs of determined concentrations of (**A**) GSH (mmol.g^{-1} of protein), obtained by HPLC-ED, (**B**) taurine (µg.g^{-1} of tissue), obtained by ion-exchange chromatography with post-column derivatization, and (**C**) metallothionein (µg.mg^{-1} of protein), obtained by differential pulse voltammetry, in samples of liver (green) and brain (blue) collected from rats fed with taurine in concentrations 0; 35; 52.5; 70; 87.5; 105; 122.5 and 140 mg.day^{-1}.

GSH is one of the markers of oxidative stress in the body [37,38]. In the rat liver the concentrations of GSH were determined in a range of 5-5.8 $mmol.g^{-1}$ of protein and in the brain in a range from 2.5 to 3.7 $mmol.g^{-1}$ of protein (Fig. 5A). In the brain and also in the liver a very small increase of GSH concentration compared with control was observed. In the liver this marker of oxidative stress increased from 5.0 ± 0.1 $mmol.g^{-1}$ of protein, measured in control sample and after feeding with 35 mg of taurine/day, to a maximum of 5.7 ± 0.1 $mmol.g^{-1}$ of protein, measured in samples from rats fed with taurine in concentration of 52.5 $mg.day^{-1}$. This concentration level remained same up to doses of 140 mg/day. In the brain, there was observed increased concentration of GSH compared to control only in rats fed with taurine in doses of 70-105 mg/day. The increase was about 0.8 $mmol.g^{-1}$ of protein from 2.9 $mmol.g^{-1}$ of protein in control to 3.7 ± 0.1 $mmol.g^{-1}$ of protein in taurine fed animals. Determined concentration levels of glutathione correspond to previously published results [33,34,39].

The concentration of taurine of 17-28 $\mu g.g^{-1}$ of tissue (Fig. 5B) was determined in the liver and in the brain approximately ten times higher, 148-215 $\mu g.g^{-1}$ of tissue, which corresponds to the values reported in the literature [40]. However, compared to Yokogoshi et al. [41], there were not observed increased concentrations of taurine in the liver, which was probably caused by lower applied concentrations. In the brain, there has been reported an increase of taurine concentration in rats fed with taurine compared to the control group, but there has not been observed increasing trend with increasing concentrations of taurine in the feed ration.

The content of metallothionein moved in both types of tissues in the range from 2.5 to 4.8 $\mu g.mg^{-1}$ of protein (Fig. 5C). This parameter may not be affected by feeding the rats with taurine, as trend in concentration changes in organs after feeding with taurine has not been observed.

4. CONCLUSION

In this work, the FIA/HPLC-ED method for detection of GSH in rat samples of brain and liver tissues was optimized. The method was used for monitoring changes in the concentration of this antioxidant compound in samples of liver and brain from rats that were fed with another antioxidant – taurine. Besides GSH, changes in the concentrations of taurine and metallothionein were monitored. The results showed a positive effect of ingested taurine on antioxidant activity in the rat liver and brain.

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Conflict of interest:
The authors have declared no conflict of interest.

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