Journal of Chromatography B, 879 (2011) 2005–2009

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

RP-HPLC method for quantitative determination of cystathionine, cysteine and glutathione: An application for the study of the metabolism of cysteine in human brain

Patrycja Bronowicka-Adamska, Jacek Zagajewski, Jerzy Czubak, Maria Wróbel*

Jagiellonian University, Collegium Medicum, Kopernika 7, 31-034 Cracow, Poland

ARTICLE INFO

Article history:
Received 13 April 2011
Accepted 16 May 2011
Available online 23 May 2011

Keywords:
Cystathionine
Cysteine
Glutathione
Human brain
RP-HPLC

ABSTRACT

The RP-HPLC method for a simultaneous separation and quantitation of the dinitrophenyl derivative of cystathionine (*N*,*N*′-di-DNP) in biological samples together with GSH, GSSG, cysteine and cystine, provides a very useful tool for investigation of the transsulfuration pathway in biological samples, at the same providing results which reflect the redox status (GSH/GSSG ratio) and the potential of the generation of H_2S. An application of the method for the study of the process of transsulfuration in various human brain regions shows the presence of cystathionine in all the investigated regions; it also demonstrates that cystathionine levels vary greatly between particular regions. The highest level in the thalamus and the lowest in the cerebellum were associated with respectively a low or high γ-cystathionase activity, and at the same time, a high cysteine and GSH level in the thalamus and a low value in the cerebellum. Based on the above results, one may suggest a regulatory mechanism responsible for inhibition of the CGL activity at high concentration values of cysteine and/or GSH. Simultaneous determinations of GSH and GSSG levels allow for determining the GSH/GSSG ratio, which reflects tissue redox status. The method may be also employed in determining the activity of γ-cystathionase and cystathionine-β synthase.

1. Introduction

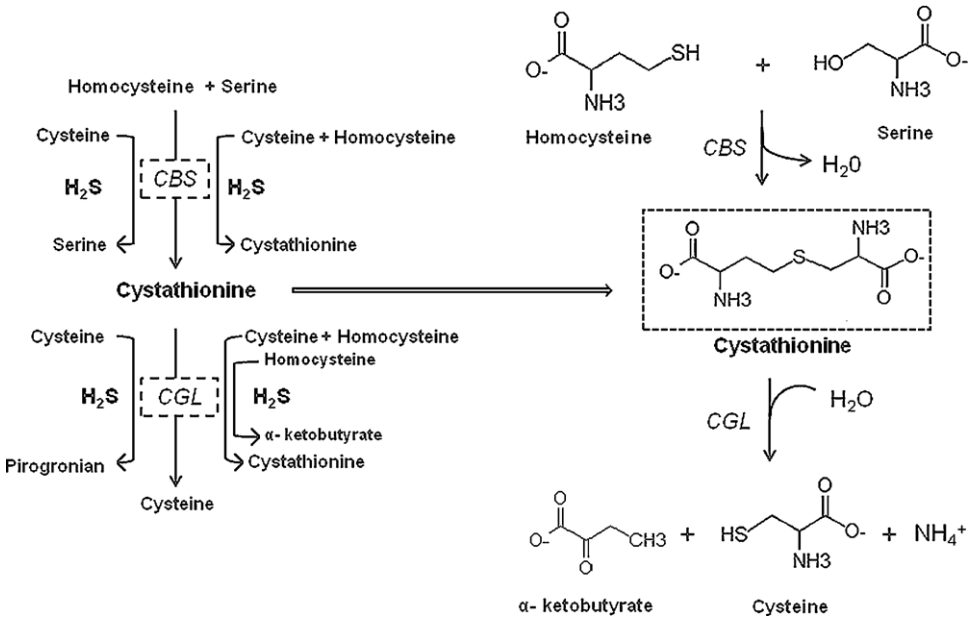
Cystathionine is an important intermediate in the L-cysteine transsulfuration pathway in mammalian tissues [1] (Scheme 1). A mean concentration of cystathionine detected in normal human serum by gas chromatographic or mass spectrometric methods equals 140 nM, with a range of 65–301 nM [2–4]. Elevated levels are found in urine of patients with neuroblastoma [5]. The level of cystathionine reflects the activity of cystathionine β-synthase ([1]CBS, EC 4.2.1.22), an enzyme responsible for cystathionine synthesis from serine and homocysteine, and cystathionine γ-lyase ([2]CGL, EC 4.4.1.1), which degrades it to cysteine, α-ketobutyrate, and ammonium ions (Scheme 1). The transsulfuration pathway is most active in such tissues as mammalian liver, kidney, pancreas, and intestine [6], but it is also present in normal brain tissue [21]. A number of reports showed the presence of cystathionine in the human brain samples collected at autopsy [7]. A relatively high level of cystathionine suggests a special relationship between the quantity and the activity of the enzymes involved in its biosynthesis and cleavage. If CGL is blocked or absent and CBS is normally active, the level of cystathionine can be elevated [6,8]. A major etiology of cystathioninuria in some neuroblastoma patients is the specific block in transsulfuration resulting from absence of CGL in the malignant tissue [5]. Cystathionine accumulates in various regions of the D,L propargylglycine-treated rat brain. The level of cystathionine in rat brain depends on the brain region. It was 10–19 times higher in the cerebellum than in the cerebral cortex and in white matter than in grey matter – this might suggests some relation of cystathionine to brain myelination [1]. In fetal brain, the concentration of cystathionine is lower than that found in the mature brain and the level increases slowly after birth until 2–3 months of age, when the value is similar to that found in the mature brain [9].

Cystathionine γ-lyase plays an important role in human brain. Diwakar and Ravindranath [10] reported that the enzyme activity was similar in most regions of the brain, except the hippocampus, where it was significantly lower as compared to cortex. The expression of CGL in all the regions of mouse and human brain as observed by *in situ* hybridization showed predominant localization in neuronal population. D,L propargylglycine inhibited the activity of CGL, what was demonstrated by the loss of [3]GSH, indicating

* Corresponding author. Tel.: +48 12 4247229; fax: +48 12 4223272.
E-mail address: mbwrobel@cyf-kr.edu.pl (M. Wróbel).

[1] Cystathionine β-synthase.
[2] Cystathionine γ-lyase.
[3] Glutathione reduced.

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.05.026



Scheme 1. Cystathionine synthesis and conversions – reactions of H_2S generation. In mammals, cysteine is made from homocysteine, which originates from methionine and supplies the sulfur atom, and serine, which supplies the carbon skeleton. The reaction is catalyzed by cystathionine-β-synthase (CBS) and yields cystathionine. In the next step, cystathionine-γ-lyase (CGL) catalyzes the removal of ammonia and cleavage of cystathionine to yield free cysteine. The two enzymes in the trans-sulfuration pathway, CBS and CGL, are believed to be chiefly responsible for H_2S biogenesis (according to Singh et al. [26]).

the importance of transsulfuration pathway in generating cysteine for GSH synthesis in [4]CNS. A significant decrease in the reducing capacity of the cellular redox couples, such as glutathione, was implicated in a number of pathologies, such as neurodegenerative disorders, epileptic seizures, demyelination (multiple sclerosis), dementia and aging [10].

The enzymatic tandem CBS/CGL is important in the production of cysteine and glutathione and also in the production of hydrogen sulfide ([5]H_2S) [11] (Scheme 1). H_2S is produced endogenously from L-cysteine in vascular smooth muscle cells and nervous system and it has a vasorelaxant property and may function as a neuromodulator. H_2S is present at a suitably high concentration in brain and CBS, which is highly expressed in the hippocampus and is involved in the production of brain H_2S [12]. In physiological concentrations, hydrogen sulfide induces the hippocampal long-term potentiation ([6]LTP), enhances [7]NMDA receptor-mediated responses and inhibits synaptic transmission in the hippocampus. The concentration of H_2S decreases in the brains of patients with Alzheimer's disease, while the overproduction of hydrogen sulfide characterizes Down syndrome patients [13].

Dominick et al. [14] developed a method for the simultaneous separation and quantitation of several thiolamines, such as glutathione reduced (GSH) and oxidized ([8]GSSG), cysteine and cystine. The procedure employs a C_{18} reversed-phase HPLC system to separate the dinitrophenyl ([9]DNP) derivatives of GSH and cysteine (*N*,*S*-di-DNP) and GSSG and cystine (*N*,*N*′-di-DNP) and relies on an internal standard, N-methyllysine, to minimize experimental error. While many methods of glutathione determination have been reported, there are a few reports concerning the simultaneous determination of GSH and GSSG [15]. We developed a modification of the method of Dominick et al. [14] allowing for the separation and quantitation of the dinitrophenyl (DNP) derivative of cystathionine (*N*,*N*′-di-DNP) in biological samples, together with GSH, GSSG, cysteine and cystine [16]. This very useful method facilitates the investigation of the transsulfuration pathway in human brain homogenates, providing results, which reflect the redox status (GSH/GSSG ratio) and the potential of the generation of H_2S.

2. Experimental conditions

2.1. Chemicals and reagents

L-Glutathione reduced, glutathione oxidized form, L-cysteine, L-cystine, cystathionine, 1-fluoro-2,4-dinitrobenzene ([10]DNFB), bathophenanthroline-disulfonic acid disodium salt ([11]BPDS), ethylenediaminetetraacetic acid ([12]EDTA), fosforan-5-pirydoksalu ([13]PLP), lactate dehydrogenase, acetonitrile were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Trifluoroacetic acid ([14]TFA) was purchased from Fluka Chemie GmbH (Buchs, Switzerland). Ethanol and 70% perchloric acid ([15]PCA) were from POCh S.A. (Gliwice). N^{ε}-methyllysine was obtained from Bachem (Bubendorf, Switzerland). All chemicals and HPLC solvents were gradient grade. Water was deionized by passing through an EASY pure RF compact ultrapure water system (MO, U.S.A.).

2.2. Methods

Various regions of human brain, collected post-mortem in the Department of Pathomorphology and Department of Forensic Medicine, Jagiellonian University Medical College, Cracow, Poland, were used in this experiment. The experimental protocol was approved by the Bioethic Commission, Jagiellonian University

[4] Central nervous system.
[5] Hydrogen sulfide.
[6] Long-term potentiation.
[7] N-methyl-D-aspartate receptor.
[8] Glutathione oxidized.
[9] Dinitrophenyl.
[10] 1-Fluoro-2,4-dinitrobenzene.
[11] Bathophenanthroline-disulfonic acid disodum salt.
[12] Ethylenediaminetetraacetic acid.
[13] Fosforan-5-pirydoksalu.
[14] Trifluoroacetic acid.
[15] Perchloric acid.

Medical College. Samples of brain (frontal cortex, parietal cortex, thalamus, hypothalamus, hippocampus, cerebellum and subcortical nuclei) were collected within 24 h of death from patients between 40 and 60 years of age if a post mortem examination performed by the attending pathologist showed that the brain was macroscopically normal. For the HPLC analysis, samples of brain were weighed and homogenized in ice-cold 10% PCA/1 mM BPDS (1 g/3 ml), during 1 min at 8000–9500 rpm using a blender homogenizer. The homogenates were centrifuged at 1400 × *g* for 10 min at 4 °C. The supernatants were used immediately or stored at −80 °C until the analysis. For CGL activity and protein determination, the brain samples were washed with cold saline and homogenized in 0.1 M phosphate buffer pH 7.5 (w/v 1:5) using a blender homogenizer (1 min, 8000–9500 rpm). The homogenates were centrifuged at 1600 × *g* for 10 min at room temperature and the obtained supernatants were used for assays.

2.2.1. HPLC instrumentation and conditions

The samples were analyzed on a 4.6 mm × 250 mm Luna C_{18} (5 μm) column with a Phenomenex Security Guard column filled with the same packing material. The chromatographic system consisted of LC-10 Atvp Shimadzu pumps, four channel degassers, column oven and a Shimadzu SIL-10 Advp autosampler. The chromatographic peaks were measured by a Shimadzu SPD-M10 Avp-diode array detector. Class VP 7.2.1 version software was used to control system operation and facilitate data collection.

A mobile phase consisting of solvent A (water/0.1% TFA) and solvent B (acetonitrile/0.1% TFA) was used for elution of samples. After injection, the column was eluted with 20% B followed with 35 min linear gradient to 55% B and 10 min isocratic period at 55% B, then 15 min linear gradient to 100% B and 10 min isocrating period. The column was then re-equilibrated to the initial conditions for 15 min. The analyses of 20 μl of each sample were performed at a flow rate of 1.0 ml/min at 20 °C temperature with diode array detection at 365 nm [16].

2.2.2. Sample preparation

The incubation mixture, total volume 456 μl, contained: 100 μl supernatant, 20 μl of 0.24 μM N^{ε}-methyllysine solution in water, 40 μl of 10% PCA/1 mM BPBS, 96 μl of 2 M KOH – 2.4 M $KHCO_3$ and 200 μl of 1% DNFB in ethanol. The samples were derivatized for about 24 h at room temperature in the dark. The reaction was stopped by adding 30μl of 70% PCA. The sample was centrifuged at 5600 × *g* for 2 min and the supernatants were filtered through a 0.20 μm PTFE Titan Syringe filter (Polygen Co., Poland).

2.2.3. Stock solutions and standard curves

Stock solutions were prepared for standard curves as follows: 2.4 μM N^{ε}-methyllysine, 1.2 μM GSH, 1.2 μM GSSG, 1.2 μM L-cysteine, 1.2 μM L-cystine, 2.2 μM cystathionine. All the stock solutions were prepared in 10% PCA/1 mM BPDS except for N^{ε}-methyllysine, which was prepared in water. A separate stock solution of the internal standard, N^{ε}-methyllysine, was prepared by dilution of 2.4 μM solution in proportion 1:10.

Standard curves were prepared in the same way as samples, by adding 20–75 μl of the stock solution of cystathionine, GSH, GSSG, cysteine and cystine instead of 40 μl 10% PCA/1 mM BPDS. Standard curves were generated in the supernatant obtained from human brain homogenate in the range from 25 to 92 nmol of each compound per ml. A linear response was shown over the concentration range investigated for all of the analytes. The linear regression analysis yielded $y = 2.28 \times 10^4 x$ ($r^2 = 0.993$) for cystathionine, where y was the peak areas (mAu) and x – the cystathionine concentration (nmol/1 ml).

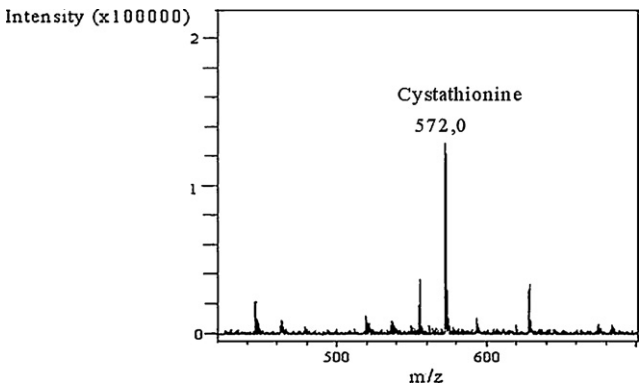


Fig. 1. The positive ionisation *m*/*z* spectrum of cystathionione.

2.2.4. Mass spectral analysis

We examined the peak areas of N,S-diDNP-cystathionine using elution gradient described in Section 2.2.1. Additionally, the fraction of cystathionine was determined by ESI/MS – the molecular ion of $m/z = 572$, $H_2O = 554$ (positive ionization M^+), corresponded to N,S-diDNP-cystathionine (Fig. 1). Mass spectrometry was performed as described elsewhere [17]. Briefly, the analyses were carried out using Esquire 3000 ESI-MS (Bruker-Daltonics, Bremen Germany) in a positive ion mode. The flow rate was set to 3 μl/min using a KD 100 Syringe pump (KD scientific, Holliston USA). Basic parameters of the ion source were as follows: heated capillary temperature: 280 °C, capillary voltage: 4.5 KV. The results were analyzed using Brukers Data Analysis software (ver 3.0).

2.2.5. Cystathionine γ-lyase assay

The CGL activity was assayed by the [18], with modifications described by Czubak et al. [19]. Each incubation mixture (650 μl) contained: 250 μl 45 mM cystathionine solution in 0.1 M phosphate buffer, pH 7.5 (10 mg of cystathionine per sample), from 275 μl to 525 μl 0.1 M phosphate buffer containing 0.05 mM 2-mercaptoethanol, pH 7.5, 25 μl 1.3 mM pyridoxal phosphate, 25 μl 13 mM EDTA–$Na_2 \cdot H_2 0$. The reaction was started by adding 75 μl of homogenate and was stopped after 30 min of incubation at 37 °C by placing 125 μl of incubation mixture in 25 μl 1.2 M perchloric acid. The samples were centrifuged at 1600 × *g* for 10 min, and 25 μl of the supernatant was transferred to 625 μl 0.194 mm NADH water solution and kept at 37 °C. The absorbance of this mixture was measured at 340 nm with a Hitachi U2000 spectrophotometer for 10 s against distilled water. After this interval, 25 μl (9.06 IU) lactate dehydrogenase from beef heart was added and the measurement was continued to 180 s [19]. The enzyme activity was expressed as pmoles α-ketobutyrate formed during 1 min incubation at 37 °C per 1 mg of protein.

2.2.6. Protein determination

Protein was determined by the method of Lowry et al. [20] using crystalline bovine serum albumin as a standard.

3. Results and discussion

The application of the modified method of Dominick et al. [14] for the study of the metabolism of low-molecular sulfur compounds in various human brain regions homogenates allows for simultaneous determinations of cystathionine, reduced and oxidized glutathione, cysteine and cystine. Examples of chromatographic distribution for homogenates of human frontal cortex, parietal cortex, thalamus, hypothalamus, hippocampus, cerebellum and subcortical nuclei are presented in Fig. 2.

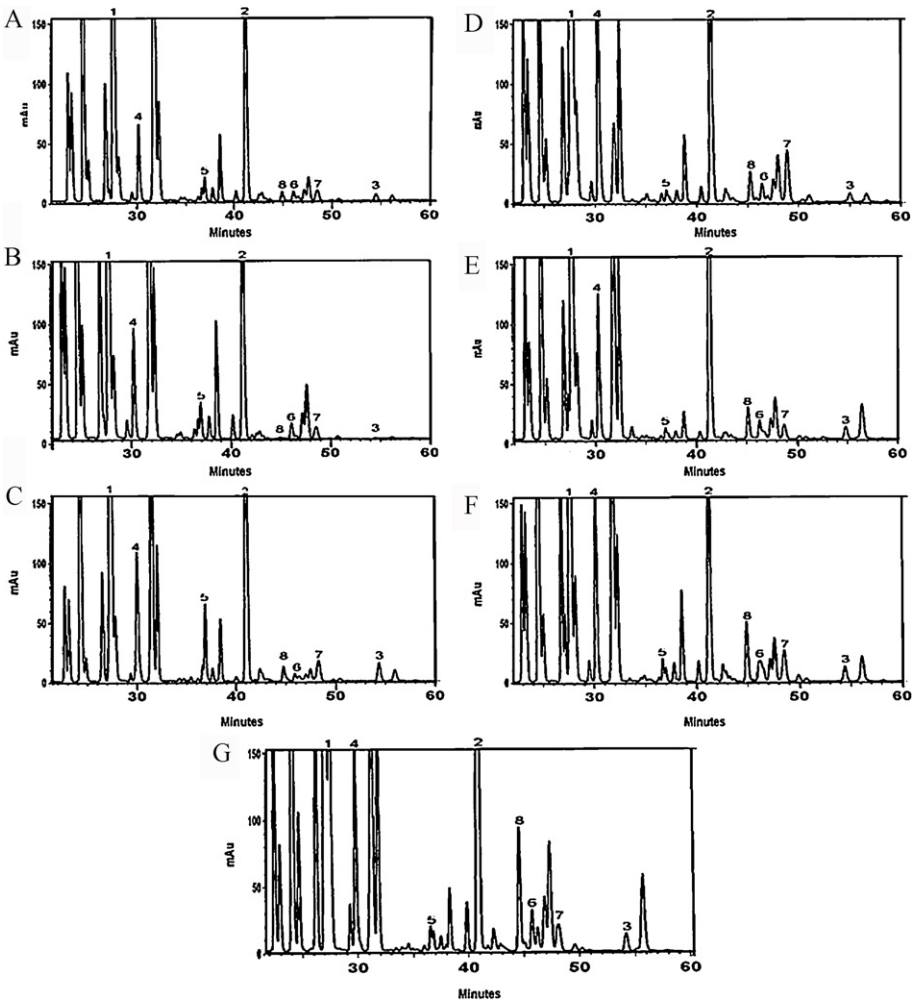


Fig. 2. Chromatograms of (A) frontal cortex, (B) cerebellum, (C) hippocampus, (D) hypothalamus, (E) parietal cortex, (F) nuclei subcortical and (G) thalamus. Peaks and retention times (min) of DNP derivatives: (1) 2,4-dinitrophenol (27.58 ± 0.24); (2) 2,4-dinitrophenyl ethyl ether (41.23 ± 0.19); (3) *N*,*N*′-di-DNP-N^{ε}-methyllysine (54.54 ± 0.27); (4) *N*,*N*′-di-DNP–GSSG (30.15 ± 0.20); (5) N,S-di-DNP–GSH (37.05 ± 0.30); (6) *N*,*N*′-di-DNP–cystine (46.08 ± 0.23); (7) N,S-di-DNP–cysteine (48.51 ± 0.28); (8) *N*,*N*′di-DNP–cystathionine (44.90 ± 0.28). Coefficient of variation (SD/mean) × 100% = 1% for all derivatives. Chromatographic conditions are described in Section 2.2.

Table 1 presents the values of GSH, GSSG, cysteine, cystine and cystathionine levels in the investigated human brain regions. Cystathionine was detected in all the investigated human brain regions and its levels vary greatly between particular regions. The level of this amino acid was the highest in the human thalamus and it was about 11 times greater than in the cerebellum ($p < 0.05$). The concentration of cystathionine in the subcortical nuclei and hypothalamus was also significantly higher as compared to the cerebellum or frontal and parietal cortex. Similarly, our research showed that the highest concentration of cysteine was noted in the thalamus, hypothalamus and subcortical nuclei (Table 1). Cysteine is the limiting substrate in biosynthesis of GSH. The highest level of cysteine in the thalamus corresponded with the highest level of GSH and the highest ratio of GSH to GSSG. The data presented in

Table 1
The level of GSH, GSSG, cysteine, cystine and cystathionine in human brain regions.

Brain region	GSH	GSSG	Cysteine	Cystine	Cystathionine
	nmol mg of protein^{-1}				
Cerebellum	0.76 ± 0.29	1.04 ± 0.55*	0.58 ± 0.21	0.23 ± 0.09	4.71 ± 2.36
Hypothalamus	0.98 ± 0.16	0.51 ± 0.38	1.73 ± 0.34	0.22 ± 0.07	23.12 ± 10.16*
Thalamus	1.49 ± 0.31	0.58 ± 0.43*	1.92 ± 0.76*	0.28 ± 0.13	55.34 ± 15.53
Subcortical nuclei	0.74 ± 0.00	1.30 ± 0.89*	1.53 ± 0.65*	0.33 ± 0.16	32.91 ± 11.03*
Hippocampus	1.28 ± 0.43	0.56 ± 0.28	0.77 ± 0.40	0.18 ± 0.08	19.79 ± 5.97*
Frontal cortex	0.54 ± 0.26	0.97 ± 0.47	0.84 ± 0.33	0.13 ± 0.08	10.27 ± 6.16*
Parietal cortex	0.42 ± 0.11	0.17 ± 0.06	0.62 ± 0.12	0.28 ± 0.02	11.88 ± 3.23
N	2–8	2–6	3–6	2–8	3–7

The values represent the mean ± SD of eight brains regions. GSH, glutathione reduced; GSSG, glutathione oxidized; CSH, cysteine; CSSC, cystine. The Mann–Whitney's test was adopted as a criterion of statistical significance with the probability level of 0.05.

* $p < 0.05$ GSSG: cerebellum, thalamus vs. subcortical nuclei; subcortical nuclei vs. frontal cortex, CSH: Thalamus vs. subcortical nuclei; subcortical nuclei vs. hippocampus and frontal cortex, cystathionine: hypothalamus vs. hippocampus and frontal cortex; subcortical nuclei vs. hippocampus and frontal cortex; hippocampus vs. parietal cortex; frontal cortex vs. parietal cortex, GSH and CSSC: statistics could not be calculated.

Table 2
Cystathionase activity in human brain regions.

Brain region	Cystathionase
pmol mg^{-1} min^{-1}	
Cerebellum	719 ± 216*
Hypothalamus	674 ± 237*
Thalamus	342 ± 114
Subcortical nuclei	555 ± 377*
Hippocampus	454 ± 222
Frontal cortex	451 ± 291
Parietal cortex	672 ± 238

The values represent the mean ± SD of eight brains, with each determination consisting of 9–15 assays. The Mann–Whitney's test was adopted as a criterion of statistical significance with the probability level of 0.05.

* $p < 0.05$ cystathionase: cerebellum, hypothalamus, subcortical nuclei vs. frontal cortex.

Table 1 allow for determining the redox status expressed by the GSH/GSSG ratio. GSH, as a reductant plays a fundamental role in the detoxification of reactive oxygen species, which is critical to the normal function of the central nervous system and its altered levels have been reported in several pathological diseases, such as schizophrenia and cancer [22–24].

A negative correlation between the amount of cystathionine and the activity of CGL was observed. The lowest among the investigated regions activity of CGL detected in the thalamus (Table 2) reflects the high level of cystathionine (Table 1). Similarly, the highest activity of γ-cystathionase was detected in the cerebellum, with the lowest among the investigated brain regions level of cystathionine. To date, the role of cystathionine in human brain it has not been confirmed, but pharmacological studies have suggested a potential role for this amino acid as a neuromodulator [25]. High cystathionine levels in some brain regions may reflect a high CBS activity. CBS is also important in the production of H_2S [11] (Scheme 1), which may function as a neuromodulator [12]. Elevated cystathionine levels might result from a low CGL activity as it was observed in case of cystathioninuria in neuroblastoma patients [5].

The method presented in this paper may be used to investigate the activity of CBS and CGL in biological samples in the presence of homoserine, CBS substrate, and in the presence of DL-propargylglycine ([16]PAG), CST inhibitor. The difference in the cystathionine level between homogenates with and without PAG can be used for the estimation of the activity of CBS in tissue homogenates. The combined activity of the CBS and CGL tandem can be measured by a difference between cysteine levels in the control and investigated samples.

4. Conclusions

The presented RP-HPLC method allows for simultaneous determinations of cystathionine, reduced and oxidized glutathione, cysteine and cystine. Cystathionine levels depend on both the CBS and CGL activities. The method produces results, which reflect tissue redox status (GSH/GSSG ratio) and the potential of the generation of H_2S and it can be also employed in determining the activity of CGL and CBS in the presence of the CGL inhibitor.

Acknowledgments

This work was supported by a grant from Collegium Medicum, Jagiellonian University, Nos. K/ZBW/000149 and K/ZDS/001468.

References

[1] S. Yu, K. Sugahara, K. Nakayama, S. Awata, H. Kodama, Metabolism 49 (2000) 1025.
[2] A.B. Guttormsen, E. Solheim, H. Refsum, Am. J. Clin. Nutr. 79 (2004) 76.
[3] S.P. Stabler, J. Lindenbaum, D.G. Savage, R.H. Allen, Blood 81 (1993) 3404.
[4] Ø. Midttun, S. Hustad, J. Schneede, S.E. Vollset, P.M. Ueland, Am. J. Clin. Nutr. 86 (2007) 131.
[5] C.E. Klein, B. Roberts, J. Holcenberg, L.M. Glode, Cancer 62 (1987) 291.
[6] J.D. Finkelstein, J. Nutr. 136 (2006) 1750S.
[7] H.H. Tallan, S. Moore, W.H. Stein, J. Biol. Chem. 230 (1958) 707.
[8] J. Wang, R.A. Hegele, Hum. Genet. 112 (2003) 404.
[9] J.A. Sturman, E.G. Gaull, W.H. Niemann, J. Neurochem. 26 (1976) 457.
[10] L. Diwakar, V. Ravindranath, Neurochem. Int. 50 (2007) 418.
[11] G. Yang, L. Wu, B. Jiang, W. Yang, J. Qi, K. Cao, Q. Meng, A.K. Mustafa, W. Mu, S. Zhang, S.H. Snyder, R. Wang, Science 322 (2008) 587.
[12] H. Kimura, Biochem. Biophys. Res. Commun. 267 (2000) 129.
[13] P. Kamoun, Amino Acids 26 (2004) 243.
[14] P.K. Dominik, P.B. Cassidy, J.C. Roberts, J. Chromatogr. B 761 (2001) 1.
[15] D.J. Reed, J.R. Babson, P.W. Beatty, A.E. Brodie, W.W. Ellis, D.W. Potter, Anal. Biochem. 106 (1980) 55.
[16] M. Wróbel, I. Lewandowska, P. Bronowicka-Adamska, A. Paszewski, Amino Acids 37 (2009) 565.
[17] M.J. Noga, J.J. Lewandowski, P. Suder, J. Silberring, Proteomics 5 (2005) 4367.
[18] Y. Matsuo, D.M. Greenberg, J. Biol. Chem. 230 (1958) 545.
[19] J. Czubak, M. Wróbel, H. Jurkowska, Acta Biol. Cracov. Ser. Zool. 44 (2002) 113.
[20] O.H. Lowry, N.J. Rosenbrough, A.L. Farr, R.I. Randall, J. Biol. Chem. 193 (1951) 265.
[21] O. Elliot, N.A. Holtzman, V.A. McKusick, Primates 15 (1974) 227.
[22] M. Terpstra, P.G. Henry, R. Gruetter, Magn. Reson. Med. 50 (2003) 19.
[23] K. Aoyama, M. Watabe, T. Nakaki, J. Pharmacol. Sci. 108 (2008) 227.
[24] Ch. Choi, Ch. Zhao, I. Dimitrov, D. Douglas, N.J. Coupland, S. Kalra, H. Hawesa, J. Davis, J. Magn. Reson. 198 (2009) 160.
[25] V. Vitvitsky, M. Thomas, A. Ghorpade, H.E. Gendelman, R. Banerjee, J. Biol. Chem. 281 (2006) 3578.
[26] S. Singh, D. Padovani, R.A. Leslie, T. Chiku, R. Banerjee, J. Biol. Chem. 284 (2009) 22457.

[16] DL-Propargylglycine.