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

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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

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

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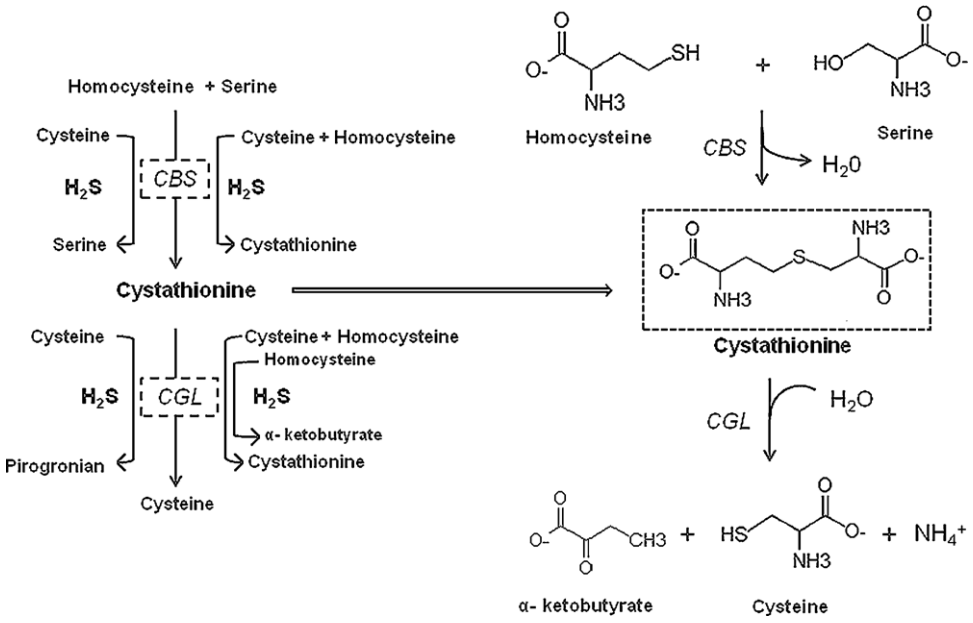
doi:10.1016/j.jchromb.2011.05.026



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.