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Analysis of Free Amino Acids in Mammalian Brain Extracts

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Abstract—An optimized method for analysis of free amino acids using a modified lithium-citrate buffer system with a Hitachi L-8800 amino acid analyzer is described. It demonstrates clear advantages over the sodium-citrate buffer system commonly used for the analysis of protein hydrolysates. A sample pretreatment technique for amino acid analysis of brain extracts is also discussed. The focus has been placed on the possibility of quantitative determination of the reduced form of glutathione (GSH) with simultaneous analysis of all other amino acids in brain extracts. The method was validated and calibration coefficient (K_{GSH}) was determined. Examples of chromatographic separation of free amino acids in extracts derived from different parts of the brain are presented.

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Amino acids are not only the building blocks in protein synthesis, but also effective regulators of biological processes including transcription and signal transduction. The results achieved in recent years in analysis of pathological changes occurring in humans under various stresses from extreme conditions showed that the fine changes in the pool of free amino acids and their metabolites are sensitive indicators of metabolic alterations [1]. In this connection, the analysis of amino acids in complex biological mixtures (tissue homogenates or extracts, physiological fluids) can be used as an indicator either in diagnostics of many diseases during the latent (asymptomatic) stages or for evaluation of therapeutic effect. Such diagnostics have been used for epilepsy to predict the disease progression and optimization of the used drugs, as well as during diagnostics of acute hereditary diseases with high mortality. Pathological changes in the synthesis and degradation of amino acids have been observed in brain injury, ischemia, neurodegeneration, malignant transformation, and other pathological conditions. These changes are the subject of intensive research.

Amino acid analysis for quantitative determination of free amino acid content in biological fluids and tissues is a two-step process. The first step is related to sample pretreatment. This is, first, the completeness of extraction using reagent(s) precipitating proteins, and the necessity of removal of other components (non-amino acids) primarily of lipid nature that interfere with the following amino acid analysis.

The second step is related to the efficiency of chromatographic separation of multicomponent mixtures and quantitative determination of individual components. In this regard, the fact that brain extracts contain many amino acids (up to 50 components) and that many other amino acids, being equally important functionally, are present as minority components increases the difficulty of brain extracts analysis.

Quantitative determination of low molecular weight thiol-containing amino acids such as cystine as well as oxidized and reduced forms of glutathione, which play an essential role in cell biogenesis, is particularly difficult.

The free amino acids in brain extracts were analyzed using an L-8800 amino acid analyzer (Hitachi, Japan) with a modified lithium-citrate buffer system [2], which offered significant advantages over the sodium-citrate buffer system used previously [3]. The study included

Abbreviations: DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; GSH, reduced glutathione; GSSG, oxidized glutathione.

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experiments on optimization of the sample pretreatment technique that is required for conducting the amino acid analysis. Special attention was paid to the possibility of quantitative analysis of the reduced form of glutathione (GSH) (the method was validated) with simultaneous analysis of all other amino acids in the brain homogenate in comparison with the colorimetric method for quantitative determination of the total content of reduced glutathione. Examples of chromatographic separation of free amino acids in brain extracts obtained from different parts of physiologically normal brain are presented in this work.

MATERIALS AND METHODS

Reagents. Analytical chromatography conditions: eluent (lithium-citrate buffers L-8500-PF-kit; Wako Chemicals GmbH, USA); ninhydrin coloring solution kit for Hitachi 299-70501 (Wako Chemicals GmbH); 5,5′-dithiobis-2-nitrobenzoic acid, research grade, Ellman's reagent (Serva, Germany); reduced glutathione (GSH; Fluka Analytical, USA); oxidized glutathione (GSSG; Serva); 2-mercaptoethanol (pure; Ferak, Germany).

Preparation of rat brain extracts. Albino Wistar or Sprague Dawley rats were kept under appropriate conditions in the vivarium at temperature 21 ± 2°C and 12/12-h day-to-night ratio. All experiments with animals were carried out according to the animal utilization protocol approved by the Ethics Committee of the Belozersky Institute of Physico-Chemical Biology (protocol No. 4, May 12, 2016). All possible measures were implemented in the study to minimize animal suffering and to use the minimum number of animals required for producing reliable data.

The rats were killed via decapitation. Brains were isolated, and cerebral cortex and cerebellum were separated and frozen in liquid nitrogen. Extracts of cerebral cortex or cerebellum were prepared using methanol and acetic acid according to a published technique [3]. Tissues stored at −70°C were homogenized in ice-cold methanol. Samples of cerebral cortex (0.4-0.5 g for Wistar rats and 0.15 g for Sprague–Dawley rats) and of cerebellum (1/2, 0.10-0.15 g for Wistar rats and 0.05-0.10 g for Sprague–Dawley rats) were placed in cooled plastic tubes of 5- or 2-ml volume (depending on weight) and weighed. Ice-cold 100% methanol was added to the cerebral cortex samples (4 ml in the case of Wistar rats and 1.5 ml in the case of Sprague–Dawley rats) and to the cerebellum samples (1 ml). The amount of methanol added during the extract preparation was calculated according to the proportion of 4 ml of methanol per 500 mg of wet tissue. Homogenization was performed using an ULTRA-TURRAX IKA T10 basic for 2 min at speed "3". One milliliter of homogenate was placed into a 5-ml tube, and 1.5 ml of 0.2% acetic acid was added and mixed with a Daigger Vortex Genie 2 (Daigger & Company, Inc., USA). Tubes were next placed into an Excella E24 shaker (New Brunswick Scientific, Germany) for mixing on ice for 30 min at 180 rpm. The samples were then homogenized in a fixed amount of ice-cold methanol independent on the tissue mass, centrifuged using an Eppendorf 5810R centrifuge for 30 min at 4°C and 4000*g*, and the samples homogenized in methanol with the specified ratio of added methanol to the tissue mass were centrifuged using a Hitachi centrifuge at 4°C and 18,000*g* for 20 min. Supernatant was collected in clean cooled tubes and stored at −70°C until used for analysis.

Amino acid analysis of samples. Amino acid analysis of the brain extracts was conducted on an L-8800 amino acid analyzer (Hitachi) with two single-channel colorimeters. Detection was carried out at two wavelengths (570 and 440 nm) according to the manufacturer's instructions. Separation was performed on an ion-exchange column packed with sulfonated styrene-divinylbenzene copolymer (type 2622SC-PF, Hitachi Ltd., P/N 855-4507, 4.6 × 60 mm) using a step-wise gradient of Li-citrate buffers (PF1-PF5) at elution rate 0.4 ml/min and temperature gradient during chromatographic separation (the column was thermostated in the temperature interval 30-70°C). A mixture of amino acid standards (AA-S-18-5ML analytical standard of acidic and neutral amino acids, Sigma; standard of basic amino acids type B, Hitachi) was used for calibration of the instrument. Aliquots of standard mixtures containing 2 nmol of each introduced amino acid were used.

On-line post-column amino acid derivatization was used for quantitative assessment of amino acids in the eluate, which occurred on mixing of the eluate with ninhydrin reagent solution supplied by a separate pump (+136°C, rate 0.35 ml/min). The ninhydrin reagent comprised special ninhydrin buffer R2 and ninhydrin reagent R1 (Wako Pure Chemical Industries; P/N 298–69601). The stained products of derivatization were detected at 570 nm for all amino acids except proline and hydroxyproline, which were recorded colorimetrically at 440 nm.

MultiChrom for Windows software (Ampersand Ltd., Russia) was used for processing the chromatographic data.

Independent of the type, the samples were centrifuged through membrane ultrafilters (centrifuge concentrators Microcon YM, 3 kDa (Millipore, USA) or Vivaspin 500 Membrane 3000 PES MWCO (Sartorius, Germany)). Aliquots (100-120 μl) were taken for amino acid analysis.

Validation of quantitative GSH and GSSG assay on the amino acid analyzer. The method was validated in accordance with the requirements of ICH International Conference (1996) using calibration curves. Model GSH and GSSG mixtures were prepared containing 0 (control), 21.5, 43, and 58 μM, respectively, and amino acid analysis of 50-μl samples was conducted under standard conditions.

Colorimetric determination of free low molecular weight thiols in brain tissue extracts using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). The concentration of free low molecular weight thiols in extracts (cerebral cortex and cerebellum) of brain tissue from the experimental animals was conducted according to Ellman's procedure [4] with the modification of Sedlak and Lindsay [5]. Only free thiol groups were recorded because the extracts were efficiently deproteinized. An aqueous 6 mM DTNB solution was used. The reaction mixture contained 0.4 M Tris-HCl buffer, pH 8.7, and 0.15 mM DTNB solution. The reaction was initiated by addition of extract to the reaction mixture. The volume of added sample ensured linear dependence of optical density on the amount of the extract. Maximum absorption at 412 nm was achieved during incubation of the mixture in dark at 25°C for 3-5 min. Measurements were conducted with a Tecan Sunrise spectrophotometer (Austria) using 0.2 ml of the reaction mixture. Background absorption was measured in the reaction mixture without extract addition; the absorption of sulfhydryl groups was obtained by subtraction of the background absorption from the total absorption of the reaction mixture. The obtained concentrations of SH groups were calculated using molar extinction coefficient (13,700 $M^{-1}\cdot cm^{-1}$) and are presented in μmol per g of wet weight of tissue.

Statistical data processing. Student's *t*-test for two data sets with unequal variances was used to evaluate significance of the differences between the results of spectrophotometric GSH assay and of its chromatographic determination with post-column derivatization. The normality of GSH content distribution determined with the two techniques was tested using the Kolmogorov–Smirnov normal distribution criterion, and then reliability of the non-zero differences between the average GSH contents was tested with the one-sample Student's *t*-test.

RESULTS

Quantitative determination of amino acids using Li-citrate buffer systems. The Hitachi model L-8800 amino acid analyzer was used in this study. The main reason for selecting this method for the study was its exceptional capability of simultaneous determination of many amino acids present in the investigated samples of tissue extracts. Separation was conducted on a 2622SC-PF chromatographic column (Hitachi) specifically designed for analysis of free amino acids in similar complex samples. The mixture is separated with elution with a stepwise gradient of buffer solutions with pH increasing from 2.8 to 4.1 and Li concentration from 0.09 to 1.0 N. Temperature gradient was used during analysis (column thermostated in the temperature interval 30-70°C). The program for stepwise eluent and temperature gradients is

Program of stepwise gradients of eluents and temperature

Time, min	PF1, % * pH 2.8 (0.09 N)	PF2, % pH 3.7 (0.255 N)	PF3, % pH 3.6 (0.721 N)	PF4, % pH 4.1 (1.0 N)	PF5, % (0.2 N)	Temperature, °C
0	100	0	0	0	0	38
2	100	0	0	0	0	30
21.6	80	20	0	0	0	60
33.5	70	30	0	0	0	
33.6	10	90	0	0	0	
36.5	10	90	0	0	0	40
43.5	10	90	0	0	0	
43.6	0	100	0	0	0	
50.5	0	100	0	0	0	70
50.6	0	0	100	0	0	
68.4	0	0	100	0	0	45
69.6	60	0	0	40	0	
75.1	0	0	0	100	0	
82	0	0	0	100	0	
82.1	0	20	0	80	0	
92.5	0	20	0	80	0	70
99.6	0	0	0	100	0	
110.6	0	0	0	0	100	
117.1	100	0	0	0	0	
123.2	100	0	0	0	0	38
127	100	0	0	0	0	

* The used eluents PF1-4 are presented, pH and ionic strength (Li^+); PF5, lithium hydroxide solution.

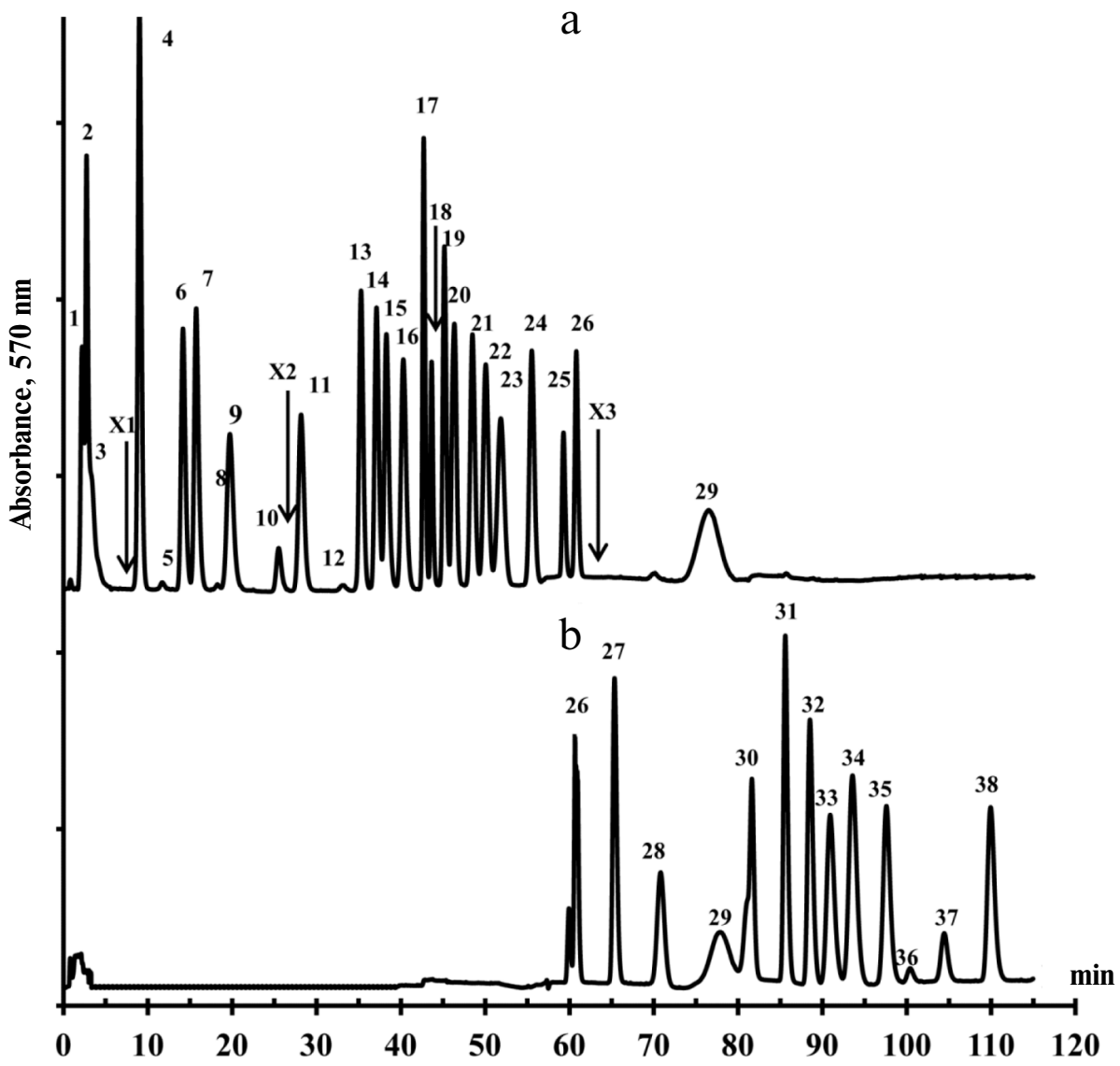

Fig. 1. Chromatographic separation of standard amino acid mixtures. a) Mixture of acidic and neutral amino acids (type AN, Hitachi, 010-08421); b) mixture of basic amino acids (type B, Hitachi, 016-08641). Amino acids are labeled with numbers: 1) phosphoserine; 2) phosphoethanolamine + taurine; 3) urea; 4) aspartic acid; 5) hydroxyproline; 6) threonine; 7) serine; 8) asparagine; 9) glutamic acid; 10) sarcosine; 11) α-aminoadipic acid; 12) proline; 13) glycine; 14) alanine; 15) citrulline; 16) α-aminobutyric acid; 17) valine; 18) cystine; 19) methionine; 20) cystathionine; 21) isoleucine; 22) leucine; 23) tyrosine; 24) phenylalanine; 25) β-alanine; 26) α-aminoisobutyric acid; 27) γ-aminobutyric acid; 28) ethanolamine; 29) ammonia; 30) oxylysine; 31) ornithine; 32) lysine; 33) 1-methylhistidine; 34) histidine; 35) 3-methylhistidine; 36) anserine; 37) carnosine; 38) arginine. Retention times of glutathione (reduced and oxidized) and homocystine that are not present in the standard amino acid mixture are denoted by X1, X2, and X3. Absorbance at 570 nm is presented; Y-axis scale is 50 mV. Aliquots of 4 nmol of all amino acids are introduced except 1, 2, 8, 11, 16, 20 (2 nmol), 10 (10 nmol), 3 (80 nmol).

presented in the table. The decrease in the chromatographic column temperature from 70 to 52°C 50 min after the start of analysis allowed better separation in the region of elution of tryptophan, ethanolamine, and ammonia. Total time of analysis was 2 h. The increase in the time for supply of buffer solutions PF4, PF1, and PF5 (table) 100 min after the start of analysis allowed better separation in the region of elution of such amino acids as anserine, carnosine, and arginine, increasing the total time of analysis to 160 min.

The chromatographic separation of standard amino acid mixtures is presented in Fig. 1 as an example. The intensity of ninhydrin staining of amino acids is characterized using color indicator (coefficient), and low indicator values are taken into consideration during the calibration of the instrument. As can be seen in Fig. 1, amino acids hydroxyproline (5) and proline (12), detected at 570 nm, are manifested only weakly because they exhibit yellow staining with ninhydrin. The amino acid analyzer is equipped with two colorimeters, one of which

allows quantitative determination of amino acids at 440 nm.

In addition to proline and hydroxyproline, cystine also forms a yellow product with ninhydrin resulting in partial decrease in color intensity at 570 nm. Nevertheless, quantitative determination of cystine on the amino acid analyzer is a well-established technique and does not present any problems (High-Technologies Corporation, Japan, 1998) [6]. The direct quantitative determination of cysteine on an amino acid analyzer is impossible due to its chemical lability. Additional derivatization must be performed for quantitative cysteine determination or its oxidation to cysteic acid by performic acid, which is considered a simpler and more reliable technique. Cysteic acid shows stable ninhydrin staining with 100% yield [7].

It also should be taken into consideration that depending on experimental conditions (slight changes of column temperature during analysis, of buffer solution pH, elution program) the intensity of color can change slightly, which is corrected during the calibration of the instrument. It should be considered during interpretation of chromatograms that the ninhydrin reagent is not strictly specific for amino acids. Being a specific agent for primary amino groups, the ninhydrin reagent nonetheless forms derivatization products with primary and secondary amides, ammonia, amino alcohols, amino sugars, and short peptides if they present in the sample. All these factors need to be considered during interpretation of the sample chromatograms.

At the beginning of our study, there was no available information on retention times and chromatographic peak shapes of reduced and oxidized glutathione. In the fundamental work of Hamilton in 1963 [8] reporting separation by elution of 186 amino acids and related compounds, several di-, tri-, and tetrapeptides, some amino sugars, amines, and amino alcohols, as well as of some other ninhydrin-positive compounds (10^{-8} M of each), reduced glutathione was not mentioned. Separation in that work was conducted using three Na-citrate buffers with pH 2.88, 3.80, and 4.30. Total analysis time was 21 h. Only the retention time of oxidized glutathione was presented in that work, which was close to the retention times of S-ethylcysteine, meso-lanthionine, and α-aminoisobutyric acid. Asymmetric shape of the peak and low intensity of ninhydrin staining engaged our attention.

As a first step in evaluation of the possibility for developing the assay of reduced and oxidized glutathione on an amino acid analyzer, we determined retention times denoted as X1 and X2, respectively (Fig. 1). Under conditions of chromatographic separation that we used for analysis of free amino acids in the rat brain extracts, reduced glutathione, which is a rather acidic tripeptide, was eluted slightly before aspartic acid and presented as an easily processed symmetric peak. Oxidized glutathione, which in fact is a hexapeptide, reacts weakly with ninhydrin (low detector output) and provides an asymmetric peak, which, as a result, does not allow its quantitative determination using amino acid analysis.

Chromatograms of rat brain extracts. The conditions suggested for chromatographic separation of amino acids were used during the investigation of the changes in activity of 2-oxo acids dehydrogenases on introduction of thiamine, which were associated with differences in metabolic transformations, and for this purpose the amino acid profiles characteristic for different brain parts were investigated in our previous work [9]. The free amino acid chromatograms of the rat brain extracts derived from two brain parts (cerebral cortex and cerebellum) are presented in Fig. 2 as an example. As seen in the chromatograms (Fig. 2, a and b), reduced glutathione denoted as X1 is easily identifiable aspartic acid before. Considering that the region of neutral amino acids is represented by several minor components, Fig. 2, a and b, has insets representing the results of analysis at higher sensitivity of the detector.

Evaluation of possibility for quantitative determination of reduced and oxidized glutathione on an amino acid analyzer. To evaluate the possibility for quantitative determination of GSH and GSSG, in the first step aqueous solutions of GSH (40 μg/ml in 0.1% β-mercaptoethanol solution) and GSSG (40 μg/ml in water) were prepared and their retention times were determined under conditions of amino acid analysis with the use of the lithium-citrate buffer elution system (Fig. 3).

Addition of β-mercaptoethanol to the solution of reduced glutathione should prevent its spontaneous oxidation. The minor ninhydrin-positive peaks with retention times up to 5 min, which can be seen on the chromatogram fragment with GSH (Fig. 3a), are also present in Fig. 3b and in the experiment without GSH (blank) and are associated only with β-mercaptoethanol. Based on this, the peaks in the GSH chromatogram associated with the blank can be excluded from consideration.

Here we have to emphasize that almost equal aliquots of GSH and GSSG (2.6 and 2.3 nmol, respectively) were taken for analysis; however, the two peak shapes and areas under peaks were strikingly different. The detector response during the GSSG analysis was very low, and the signal-to-noise ratio was too low for quantitative determination of GSSG in the required concentration range.

The possibility for quantitative determination of GSH and GSSG contents on the amino acid analyzer was evaluated from calibration curves. The model mixture contained 0 (blank), 21.5, 42.95, and 57.92 μM GSH (Fig. 3b). The linear approximation of the area under the GSH peaks demonstrates $R^2 = 0.997$ and the intercept comprising 4% of the lower boundary of the analytical region (6.6 μg/ml = 21.5 μM). The signal-to-noise ratio (below 10) allows estimation of the limit of quantitative determination of ~0.1 μg GSH (0.325 nmol/50 μl or 6.5 μM). The value of the GSH peak area per nmol was

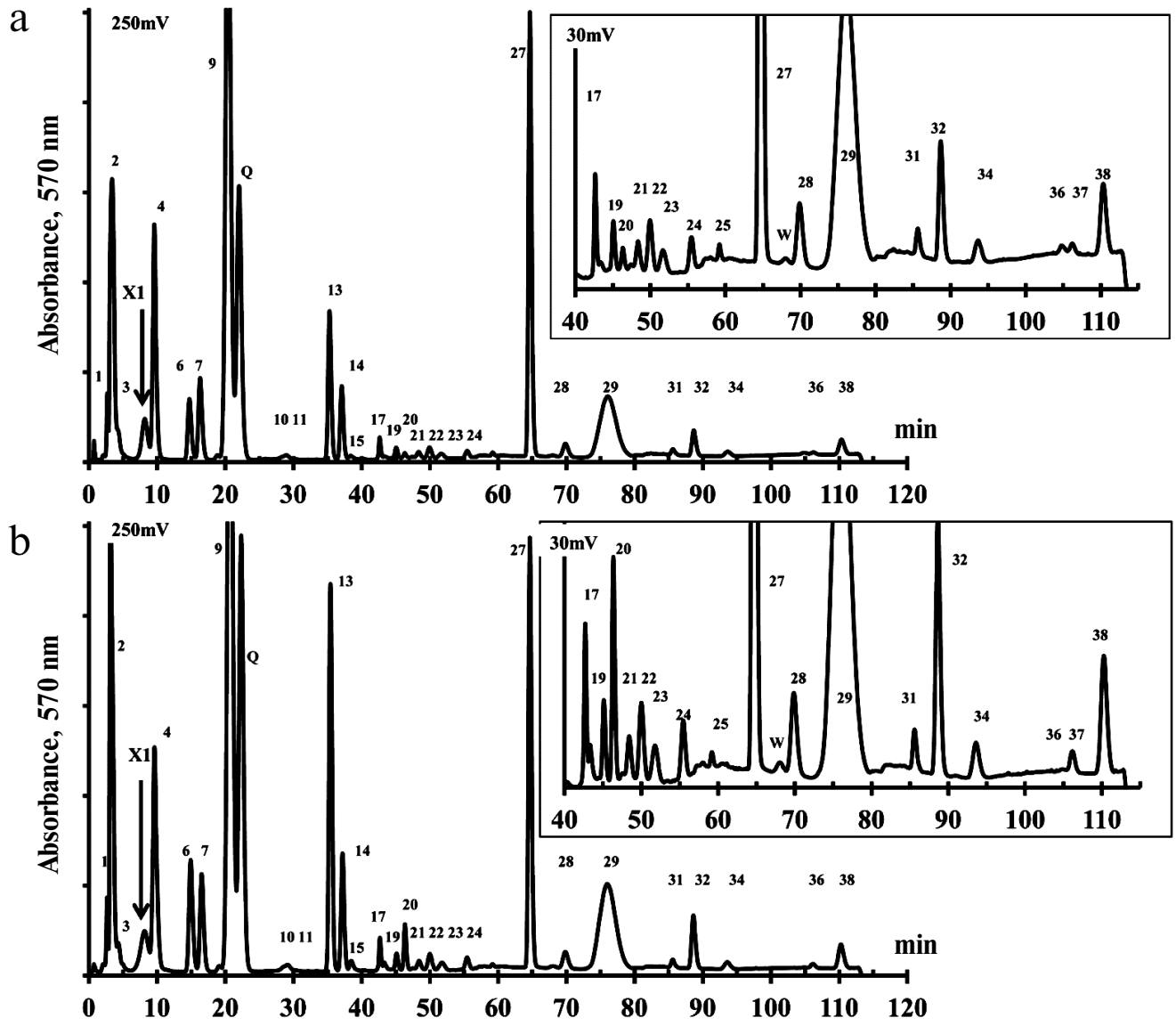

Fig. 2. Chromatographic separation of amino acids in extracts of rat brain tissues. a) Extract of 0.25 mg of cerebral cortex; b) extract of 0.17 mg of cerebellum; 110 µl of each introduced (procedure in "Materials and Methods" section). Absorbance at 570 nm is presented. Y-axis scale – 250 mV. Insets – fragments of chromatograms in region of 40-115 min, Y-axis scale – 30 mV. Numbers above peaks denotes amino acids (see Fig. 1); X1, retention time of GSH; Q, of glutamine, W, of tryptophan.

calculated from the slope of the straight line, S(GSH) = 188 mV·s/nmol (Fig. 3c). The same parameter was calculated simultaneously for most of the amino acids in the standard mixture, S(AA) = 588 ± 50 mV·s/nmol. Hence, the conversion coefficient is K = S(AA)/S(GSH) = 3.13.

The validation experiments for quantitative determination of GSSG content showed that the detector response was not sufficient, and the signal-to-noise ratio too low for quantitative determination of the GSSG content in the required concentration range, which made its determination using the amino acid analyzer impossible.

Comparison of methods for determination of GSH concentration in cerebral cortex of rat brains using DTNB and amino acid analysis. Considering that the reduced glutathione is the main thiol in a cell, the spectrophotometric method based on application of the Ellman's reagent is widely used for the assessment of free cellular thiols [5, 10]. This method is relatively simple, reliable, and does not require costly scientific instrumentation. Moreover, in many cases, the accurate estimate of this parameter is sufficient to evaluate the redox state of the cell, and total information on the quantities of other amino acids is not needed. We compared the results of spectrophotometric analysis of the GSH content in the rat cerebral cortex and cerebellum and its determination using the amino acid analyzer (Fig. 4).

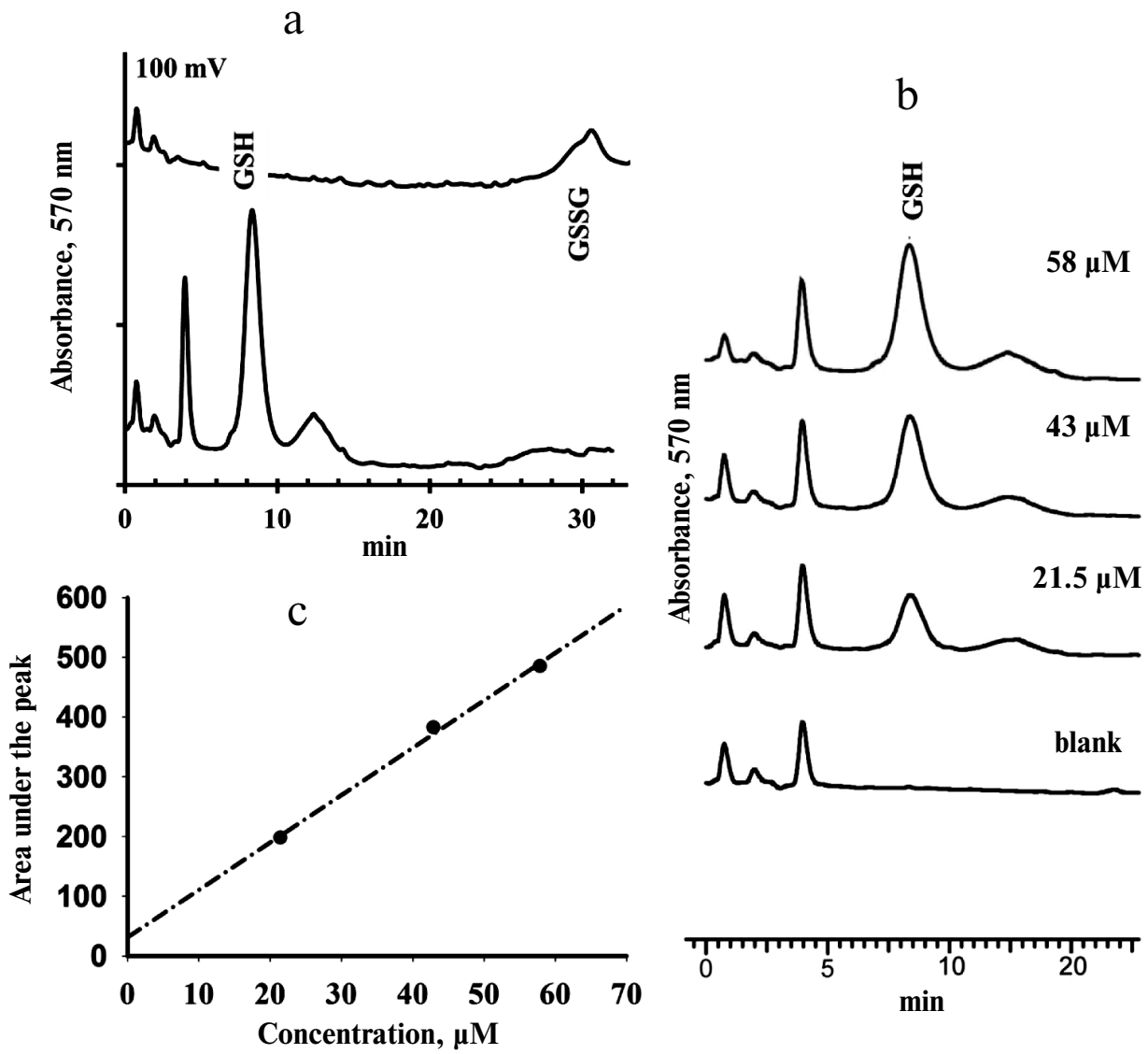

Fig. 3. a) Fragments of chromatograms of separation of model mixtures of GSH in 0.1% β-mercaptoethanol and GSSG performed on the Hitachi L-8800 amino acid analyzer using the lithium-citrate buffer system; b) calibration chromatograms of reduced glutathione in solution (absorption at 570 nm, Y-axis scale – 50 mV); GSH concentrations are indicated above the line; c) calibration dependence of area under the GSH peak on its concentration in the solution.

The two methods provide very similar values for the reduced form of glutathione: approximately 1.8 μmol/g of tissue for the cerebral cortex and approximately 1.6 μmol/g of tissue for the cerebellum. Hence, for both parts of the brain, the GSH content determined either spectrophotometrically or on the amino acid analyzer was the same (no statistically significant differences).

The Bland–Altman plot is commonly used to test the agreement of the results obtained by two different methods [11, 12]. *A priori* a 95% confidence level was set, which could be explained as follows: if 95% of results are in this confidence interval, then the methods can be considered in good agreement and one can be replaced by the other. The Bland–Altman plot representing the dependence of differences between the values produced by the two methods on the mean value for each sample [11] allows evaluating how regularly the results of both methods are in the same agreement interval (Fig. 5).

The limits of agreement between the two considered methods are presented in the Bland–Altman plot (Fig. 5) and it is shown that the mean difference between the GSH concentrations differs from zero (bold line). This means that on average the values of concentration of this tripeptide in the samples obtained with the DTNB method will be by 0.19 lower than the values determined using the amino acid analyzer. This was confirmed by the one-sample Student's *t*-test. For example, considering that the level of GSH in the rat cerebral cortex is

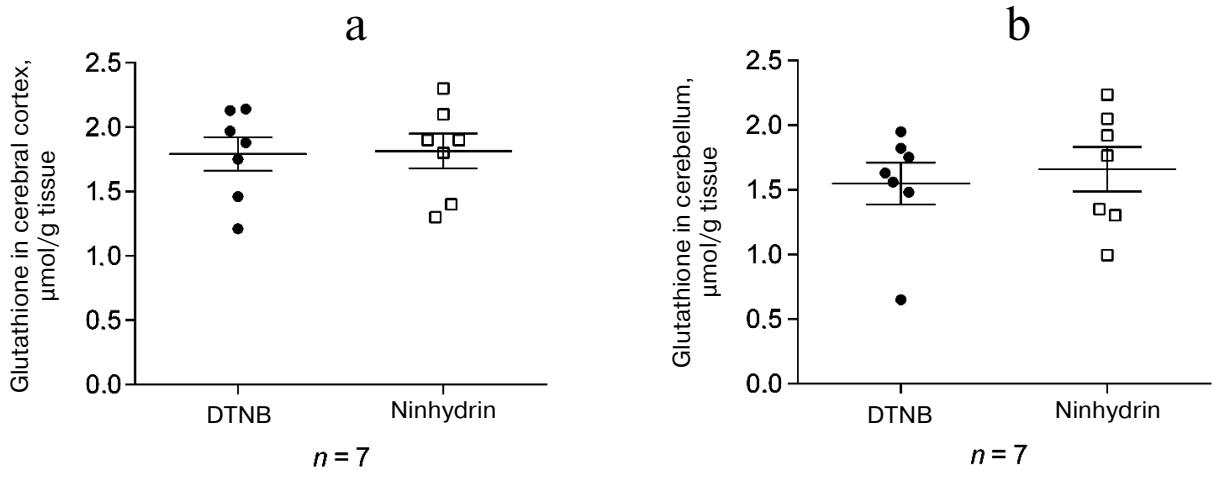

Fig. 4. Concentration of reduced glutathione in extracts of rat cerebral cortex (a) and cerebellum (b) measured with DTNB and by amino acid analysis. The data are presented as means (middle line) ± standard error of the mean (upper and lower lines), *n* – number of experimental animals.

~1.8 μmol/g of wet tissue, the difference of 0.19 corresponds to ~10% of the GSH content. Hence, the Bland–Altman plot demonstrates that the values obtained by the DTNB method can be lower by 1.17 μmol/g (maximum) and higher by 0.8 μmol/g of tissue than the GSH content evaluated from the data of an amino acid analyzer (Fig. 5).

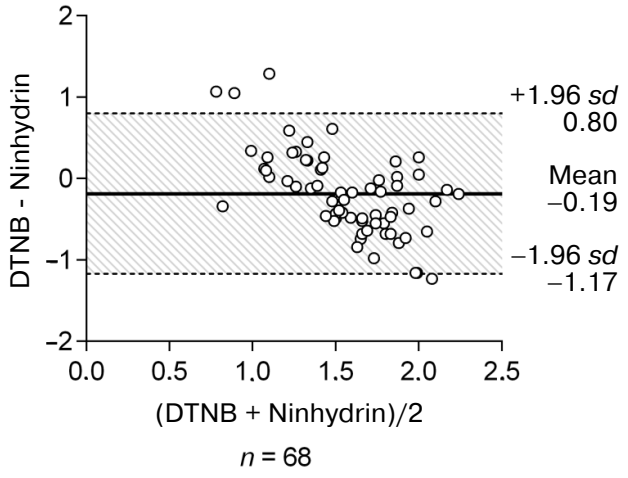

Fig. 5. Bland–Altman plot to assess the agreement between the results obtained with the method based on DTNB and by chromatographic analysis with post-column ninhydrin derivatization. Y-axis – difference between GSH concentrations obtained with these methods for one sample, X-axis – mean values of GSH concentrations obtained with these methods for each sample. Mean difference between the measurements is represented by bold horizontal line, *sd* – standard deviation of the GSH concentration differences, scatter region is marked by dashed line (from mean difference of –1.96 *sd* to mean difference +1.96 *sd*), *n* – number of experimental animals for which GSH concentrations were determined.

Thus, the results of these two methods are in good agreement considering the pre-set 95% confidence level of interval of differences, which indicates according to [13] the absence of systemic differences between these two methods.

Two more factors should be taken into account when the inconsistencies between the results of the GSH content determination are considered in more detail: first, the colorimetric method allows determination of the level of all non-protein thiols in the samples. The reduced form of glutathione is the major component of thiols in cells; however, its quantity may vary depending on the tissue and physiological conditions [14]. Second, the conditions for conducting chromatographic analysis could cause additional oxidation of thiols, primarily of reduced glutathione, which, in turn, could result in certain discrepancies with the results of spectrophotometric analysis.

DISCUSSION

Quantitative determination of free amino acid content in biological fluids and tissues using the technique of amino acid analysis is not a simple task despite the extreme stability and reliability of the method. The first problem is related to the correct sample pretreatment procedure. This involves in the first place the completeness of protein extraction with the use of special precipitating agents, and the necessity to remove impurities primarily of lipid nature, which interfere with the following amino acid analysis. In this regard, the procedure for pretreatment of free amino acid samples from brain extracts present significant problems due to the presence of large amounts of lipids of different nature. It required many

efforts before we selected conditions for maximum removal of lipid components of the brain. However, from our experience, even small amount of lipids presented in the samples prepared for analysis reduces chromatographic quality of the column rather fast, which is manifested by the broadening of amino acid peaks. Even though the "lifetime" of column was approximately 700 analyses, we were able to analyze reliably only ~500 such samples.

The second problem arising during analysis of brain extracts is related to the necessity of efficient separation of multicomponent mixtures of amino acids in which some amino acids are present in large quantities while many others no less important functionally are only minor components. The suggested conditions for chromatographic separation of free amino acids were successfully used among others in the studies of V. I. Bunik and coauthors devoted to investigation of natural and synthetic regulators of amino acid degradation for pharmacological correction of various pathologies of humans and other animals [9].

Analysis of sulfur-containing amino acids and their metabolites is in general an essential area of amino acid analysis, and it is of special importance in the analysis of free amino acids in brain tissue extracts. The biological thiol-containing amino acids are usually present as minor components in the mixtures of free amino acids derived from tissue extracts, and, hence, significant efforts are needed for their effective chromatographic separation. A shift of redox potential of thiol-containing amino acids during the sample pretreatment cannot be excluded, which could lead to deviations in the content values during their determination. The reduced and oxidized forms of glutathione occupy a special place in cell biogenesis, and we concentrated most attention on this problem in our study.

Glutathione (γ-glutamyl cysteinyl glycine) is an important tripeptide, and its intracellular concentration is an indicator of oxidative stress. Glutathione exist in two forms in cells: the reduced sulfhydryl form (GSH) and glutathione disulfide (GSSG) – the oxidized form. GSH is the predominant form. More than 95% of the "total" glutathione in tissues is in the GSH form. Considering that GSH can be easily oxidized non-enzymatically and, in addition, is a good substrate for γ-glutamyl transpeptidase, as well as GSSG, special requirements must be imposed upon preparation and storage of samples. Biological samples should be acidified very quickly to decrease the risk of GSH oxidation to GSSG and formation of mixed disulfides and to inactivate the abovementioned enzyme [15]. The optimal procedure for the biological sample pretreatment depends on the investigated tissue and on the experimental system. Tissues with high γ-glutamyl transpeptidase content must be treated as fast as possible, while tissues with low level of this enzyme such as tissues of different brain parts can be frozen to –20°C and stored for 10-20 h.

Wide availability of glutathione in cells and its obvious involvement in many biological processes generate continuous interest in the development and improvement of methods of analysis of this cellular component even though it was discovered and isolated more than 50 years ago. Oxidative stress causes significant disruption of the balance of cellular thiols and can lead to decrease in the GSH/GSSG ratio in many organs of the body. This requires accurate assessment of changes in the GSH/GSSG ratio under various physiological and pathophysiological states.

Numerous methods for determination of glutathione in biological fluids have been described: enzymatic, colorimetric, and chromatographic. It was not our goal to provide deep analysis and compare different methods for quantitative glutathione determination. One of the first issues that attracted our attention was the fact that the reason for different levels of this compound in the control and pathological samples was associated with the methods of determination of its content and variations during the sample pretreatment. Many of these methods present only historical interest and are replaced by not only faster but also significantly more sensitive techniques.

The variety of high efficiency liquid chromatography techniques allows the possibility to determine both forms of glutathione (GSH and GSSG) with high specificity at even picomolar concentrations. Furthermore, these methods provide an advantage allowing determination of various thiols and disulfides other than GSH. These methods are described in works [16, 17]. The significant drawback of these techniques, however, which reduces their applicability, is the necessity of prior derivatization of samples before the chromatographic separation and analysis. This involves either treatment with 2,4-dinitrofluorobenzene as in [16] or with monobromobimane as in [17] followed by fluorescence detection.

Another drawback of this approach is significant. Variations in quantitative determinations of both forms of glutathione are quite often due to alkalization and slow derivatization of the samples, which are required steps in the high efficiency liquid chromatography conducted prior to the sample injection. In the process, low recovery of GSH (20-80%) is often observed with extremely high recovery of GSSG [16]. These complications were partially alleviated by Rahman et al. [18]. In this study, the authors used sulfosalicylic acid not only for the medium acidification during the sample preparation, but also for inhibition of γ-glutamyl transferase, which caused the loss of GSH due to its consumption in detoxification reactions.

For quantitative determination of the reduced and oxidized forms of glutathione in rat brain extracts, we selected a classic technique of quantitative amino acid analysis on an ion-exchange column in the mode of analysis of free amino acids using lithium-citrate buffer systems and post-column derivatization with ninhydrin

for colorimetric determination of amino acids in the eluate [6]. Retention times of both forms of glutathione were determined, which allowed us to hope for the possibility of their quantitative determination simultaneously with other amino acids in the rat brain extracts. In the separate experiments, validation of the suggested technique was conducted, which demonstrated that quantitative determination on the amino acid analyzer was possible only for the reduced form of glutathione. Quantitative determination of the oxidized glutathione in the required concentration range was impossible due to the low detector response, and the signal-to-noise ratio was too low for quantitative determination of oxidized glutathione in the required concentration range. As an alternative, the spectrophotometric method based on the use of Ellman's reagent was selected for assessment of free cellular thiols in the brain tissue extracts. The data of amino acid analysis of the reduced glutathione in the extracts of rat cerebral cortex and cerebellum were compared with the data obtained for these samples using DTNB (Ellman's reagent). In general, the results of the two techniques are in good agreement in the pre-set confidence interval, because the average difference is in the 95% interval of differences, which indicates the absence of systemic differences between these two techniques.

The conditions for conducting amino acid analysis of free amino acids in rat brain extracts using an amino acid analyzer (model L-8800, Hitachi) were optimized in this work. Separation of the mixture of amino acids was carried out by elution using stepwise gradient of lithium-citrate buffer solutions. The possibility of quantitative determination of the reduced form of glutathione simultaneously with analysis of all other amino acids in the brain homogenate was demonstrated.

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