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An alternative derivatization method for the analysis of amino acids in cerebrospinal fluid by gas chromatography–mass spectrometry

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ABSTRACT

The determination of the concentrations of L-amino acids in cerebrospinal fluid (CSF) has been used to gain biochemical insight into central nervous system disorders. This paper describes a microwave-assisted derivatization (MAD) method using N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) as a derivatizing agent for determining the concentrations of L-amino acids in human CSF by gas chromatography with mass spectrometry (GC/MS). The experimental design used to optimize the conditions showed that the optimal derivatization time was 3 min with a microwave power of 210 W. The method showed good performance for the validation parameters. The sensitivity was very good, with limits of detection (LODs) ranging from 0.01 μmol L^{-1} to 4.24 μmol L^{-1} and limits of quantification (LOQs) ranging from 0.02 to 7.07 μmol L^{-1}. The precision, measured using the relative standard deviation (RSD), ranged from 4.12 to 15.59% for intra-day analyses and from 6.36 to 18.71% for inter-day analyses. The coefficients of determination (R^2) were above 0.990 for all amino acids. The optimized and validated method was applied to the determination of amino acid concentrations in human CSF.

1. Introduction

Quantitative measurements of metabolites in biological systems have an important role and have been applied to the diagnosis of diseases, such as diabetes, cancer, Alzheimer's disease, and Parkinson's disease [1–7]. Among these molecules are amino acids [8,9]. The presence and levels of free amino acids (FAAs) in body fluids can be early indicators of neurological disease [6,10] and can be used to diagnose inborn errors of metabolism [11].

The analysis of amino acids in body fluids is often performed using chromatographic methods. In the reference method, amino acids are analyzed by ion-exchange liquid chromatography followed by post-column ninhydrin derivatization and UV detection [12]. Methods described more recently include HPLC with photodiode array and fluorescence detection [13], liquid chromatography–tandem mass spectrometry (LC–MS/MS) with electrospray ionization [8,14–16] and ultra-high-performance-liquid-chromatography–tandem mass spectrometry (UPLC–MS/MS) [11,17]. However, analysis by liquid chromatography can be time-consuming [17] and expensive [14], and the sensitivity is often low [11].

Gas chromatography with mass spectrometry detection is regarded as the gold standard in metabolite analysis due to the technical advantages, such good separation, high sensitivity, robustness, and large spectrum libraries [3,18,19]. Whenever the metabolites of interest are nonvolatile in the original sample, sample derivatization is required [20–23]. When analyzing amino acids by GC/MS, several reagents are used for derivatization, including methyl chloroformate, heptafluorobutanol, and trifluoroacetic anhydride [19,22,24].

Silylation is commonly used to analyze metabolites by GC/MS, and pyridine, which is used as an acid scavenger, is often added before the silylating agent [9,25,26]. The silylation of amino acids involves the simultaneous reaction of the amino and carboxyl groups in a single step, and when a microwave is used for heating, the reaction time significantly shorter. In comparison with derivatization using conventional heating, derivatization using microwave heating has a better relative response ratio and results in fewer artifacts in the analysis of compounds such as amino acids, sugars, and fatty acids [9]. There are few gas chromatography–mass spectrometry (GC/MS) methods described for the analysis of amino acids in biological fluids using silylating reagents [27,28]. The GC/MS methods described in the literature using other derivatizing reagents

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have high resolutions; however, glutamic acid and tryptophan, which are important in the diagnosis of neurological disorders, are not detected [22], and serine and threonine cannot be measured reliably due to secondary interactions with these analytes [14].

The aim of the present study was to develop and validate a reliable and robust alternative gas chromatography/mass spectrometry method based on a simple procedure of extraction and microwave-assisted derivatization (MAD) for the quantification of 16 amino acids, including glutamic acid, serine, and threonine, in cerebrospinal fluid and others biological fluids. This method could be used in metabolomics technologies.

2. Experimental

2.1. Chemical and materials

The following amino acids were obtained from Sigma–Aldrich (St. Louis, MO, EUA): L-alanine, L-glycine, sarcosine, L-valine, L-leucine, L-isoleucine, L-serine, L-threonine, L-aspartic acid, L-methionine, L-proline, L-cysteine, L-glutamic acid, L-phenylalanine, L-asparagine and L-lysine. HPLC-grade methanol was obtained from Merck KGaA (Darmstadt, Germany), and BSTFA (*N*,*O*-bis(trimethylsilyl)trifluoroacetamide) + TMCS (trimethylchlorosilane) (99:1 v/v) and anhydrous pyridine (99.8% m/m) were purchased from Sigma–Aldrich (St. Louis, MO, EUA). Methoxyamine hydrochloride (98% m/m) was obtained from Fluka (St. Louis, MO, EUA). Artificial cerebrospinal fluid (aCSF) containing 127 μmol L^{-1} NaCl, 2 μmol L^{-1} KCl, 1.2 μmol L^{-1} KH_2PO_4, 26 μmol L^{-1} $NaHCO_3$, 2 μmol L^{-1} $MgSO_4$, 2 μmol L^{-1} $CaCl_2$, 10 μmol L^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 10 mmol L^{-1} glucose and bubbled with a carbogenic mixture (95% v/v O_2 and 5% v/v CO_2) was prepared. All reagents were obtained from Sigma–Aldrich (St. Louis, MO, EUA). The aCSF was stored at 4 °C and prepared weekly. The standard solutions for each amino acid were prepared at an initial concentration of 1.0 mg mL^{-1} in H_2O and stored at −20 °C. Primary standard mixtures with a concentration of 10.0 μg mL^{-1} were prepared from the separate standard solutions in H_2O to optimize the microwave-assisted derivatization (MAD) procedure. These working solutions were stored at 4 °C.

2.2. Sample preparation and derivatization process

A sample of 200.0 μL of aCSF was transferred to an Eppendorf tube, and 800.0 μL of methanol at −10 °C was added to precipitate the protein. The extraction procedure was adapted from previously described methods [29]. The solution was vortex mixed for 1 min and centrifuged at 10,000 rpm for 10 min at room temperature. Then, 200.0 μL of supernatant was transferred to a glass GC vial and evaporated to dryness at room temperature under N_2. Next, 15.0 μL of methoxyamine in pyridine at 20.0 mg mL^{-1} was added, followed by 35.0 μL of BSTFA with TMCS. The solution was vortexed for 30 s and submitted to microwave irradiation in a domestic microwave oven (700 W power) equipped with a turning table. In all experiments, the vial was always placed at same point to ensure even irradiation. Initially, a set of experiments using varied power operations (high, medium and low) and different heating durations was performed to evaluate the response, reproducibility, and speed of the analysis using MAD.

2.3. Sample collection

A total of 16 CSF samples were obtained from hospitalized patients in Santa Casa de Misericórdia de BH (Belo Horizonte, Brazil). Patients presents suspect of bacterial infection (cryptococcal meningitis) with characteristic symptoms and waiting

Table 1
Analytical characteristics of L-amino acid derivatives using select ion monitoring (SIM).

No	Amino acids	Retention time (min)[a]	Identification ions (*m/z*)	Quantitation ions (*m/z*)	Linearity (R^2)	LOD[a] (μmol L^{-1})	LOQ[a] (μmol L^{-1})	Recovery[b] (%)	Precision[c] (%)	
									Inter	Intra
1	L-Alanine	7.64 ± 0.02	116(100) + 73(53.6)	116	0.9947	0.38 ± 0.02	0.64 ± 0.03	102 ± 5	11.39	5.08
2	Glycine	7.42 ± 0.01	102(100) + 73(68.4)	102	0.9906	0.40 ± 0.02	0.66 ± 0.03	110 ± 5	15.46	4.41
3	Sarcosine	7.86 ± 0.02	116(100) + 73(52)	116	0.9934	0.33 ± 0.02	0.54 ± 0.03	98 ± 6	18.71	6.08
4	L-Valine	9.21 ± 0.02	144(100)+ 73(40.6)	144	0.9927	0.18 ± 0.01	0.30 ± 0.02	115 ± 7	13.08	6.36
5	L-Leucine	10.13 ± 0.02	158(100) + 73(42.4)	158	0.9900	0.06 ± 0.01	0.10 ± 0.01	121 ± 14	16.46	11.98
6	L-Isoleucine	10.53 ± 0.02	158(100) + 73(50.4)	158	0.9939	0.05 ± 0.01	0.09 ± 0.01	117 ± 12	17.74	10.11
7	L-Serine	11.64 ± 0.02	73(100) + 204(75.2)	204	0.9917	0.06 ± 0.01	0.10 ± 0.01	125 ± 9	19.06	7.07
8	L-Threonine	12.09 ± 0.02	73(100) + 218(46.0)	218	0.9931	0.36 ± 0.01	0.60 ± 0.02	128 ± 5	17.07	4.12
9	L-Methionine	14.16 ± 0.02	176(100) + 128(60.0)	176	0.9976	0.12 ± 0.01	0.20 ± 0.02	126 ± 5	18.48	8.61
10	L-Aspartic acid	14.18 ± 0.02	73(100) + 232(81.2)	232	0.9928	0.10 ± 0.01	0.17 ± 0.01	123 ± 11	17.06	4.19
11	L-Proline	14.25 ± 0.02	156(100) + 73(53.6)	156	0.9915	1.05 ± 0.12	1.74 ± 0.20	119 ± 14	6.36	6.09
12	L-Cysteine	14.71 ± 0.02	73(100) + 220(88.8)	220	0.9750	2.43 ± 0.36	4.04 ± 0.60	128 ± 16	18.22	15.04
13	L-Glutamic Acid	15.63 ± 0.03	246(100) + 128(32.4)	246	0.9955	0.010 ± 0.001	0.02 ± 0.01	129 ± 9	17.88	7.58
14	L-Phenylalanine	15.77 ± 0.02	218(100) + 192(71.2)	218	0.9911	0.08 ± 0.01	0.13 ± 0.01	121 ± 8	18.13	6.48
15	L-Asparagine	16.43 ± 0.03	116(92.40) + 231(51.2)	231	0.9934	0.12 ± 0.02	0.19 ± 0.03	97 ± 14	17.78	15.30
16	L-Lysine	16.80 ± 0.03	84(100) + 156(55.6)	156	0.9957	4.24 ± 0.60	7.07 ± 0.96	88 ± 12	17.58	15.59

[a] Mean ± standard deviation (n = 10).
[b] Mean ± standard deviation (n = 5).
[c] Relative standard deviation (n = 5).

Table 2
Experimental 2^3 two-level full factorial and Doehlert design for L-amino acid extraction.

Design	Variables	Low level	High level	Significance
Fatorial 2^3	Derivatization time (min)	2.0	4.0	Yes
	Microwave power (W)	140 (20%)	280 (40%)	Yes
	Amount derivatization reagent (μL)	40	100	No
Doehlert	Derivatization time (min)	2; 2.5; 3; 3.5; 4		
	Microwave power (W)	140 (20%); 210 (30%); 280 (40%)		

laboratorial diagnostic to effective suppressive therapy. The patients were screened in accordance with the guidelines established by the research ethics board (protocol N 082/2010). All samples were stored at −80 °C until use or sample preparation [30].

2.4. Gas chromatography mass spectrometric analysis

The amino acid analysis was performed using a Shimadzu (Kyoto, Japan) model GC-2010/QP-2010 high-performance quadrupole GC/MS system. The mass spectrometer was operated in electron impact (EI) mode at 70 eV. Separation of the analytes was performed on a Restek (Bellefont, PA, USA) chemically bonded Rtx-5MS fused-silica capillary column (30 m × 0.25 mm id × 0.25 μm film thickness) containing 5% diphenyl/95% dimethylpolysiloxane. The oven temperature program was as follows: 80 °C, ramp to 200 °C at 8 °C min^{-1}, ramp to 300 °C at 30 °C min^{-1}, and hold at 300 °C for 3 min. The injector was operated at 280 °C in splitless mode for 3 min, followed by a 1:20 split ratio (RD). Helium with a purity of a 99.999% was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. Samples were injected manually with a 10.0 μL Hamilton micro syringe (Reno, NV, USA) in splitless mode with a 1 minute vent open time, and a volume of 1.0 μL was injected. The ion source temperature was 200 °C, and the GC/MS interface was kept at 260 °C. For identification and confirmation, each amino acid solution was prepared separately for derivatization under the optimized conditions. A 1.0 μL aliquot was injected into the GC/MS, and the analysis was performed in full scan mode (range 45–300 *m/z*) with a scan time of 2 scans s^{-1}. The retention times achieved and the ions that were used for identification and quantification are shown in Table 1. The quantification was performed using single ion monitoring (SIM). Signal acquisition and data processing were performed using the Shimadzu LabSolutions software (Kyoto, Japan). A domestic Philco model PRC-700W microwave (São Paulo, Brazil) was used for the MAD.

2.5. Experimental design

For the optimization step, all the experiments were performed with aCSF. A study was conducted using a 2^3 two-level full factorial design (FFD) to investigate the following variables: derivatization time, microwave power, and amount of derivatization reagent. The experimental values of these variables are presented in Table 2. The minimum and maximum levels of the parameters were selected based on protocols that use MAD [13,24]. The Doehlert design (Table 2) was applied using the most significant variables with the objective of reaching the optimum region of the investigated area. When applied to two variables, the Doehlert design requires performing seven experiments [29]. The experimental response (Y) as a function of the variables (X_1 and X_2) is given by Eq. (1):

$$Y = b_0 + b_1(X_1) + b_2(X_1)(X_1) + b_3(X_2) + b_4(X_2)(X_2) + b_5(X_1)(X_2) \quad (1)$$

where b_0 is the constant term, b_1 and b_3 are the coefficients for the linear terms, b_2 and b_4 are the coefficients for the quadratic terms, and b_5 is the coefficient for the interaction between the two factors. The fitting of the model was validated using analysis of variance (ANOVA). *P*-values smaller than 0.05 were considered significant. However, because this procedure used multiresponse tests, it was necessary to use the desirability function to obtain a simultaneous and satisfactory compromise between all the responses. This approach is defined as the geometric mean of the individual desirabilities. The desirability function transforms an estimated response into a scale-free value, called the desirability (d_i). The desirability is a value between 0 and 1 and increases as the corresponding response value becomes more desirable [30]. The overall desirability (D), another value between 0 and 1, is the geometric mean of the individual desirability values (d_i):

$$D = (d_1 d_2 \dots d_n)^{1/n} \quad (2)$$

Then, the optimal setting is determined by maximizing D. All the statistical analyses were performed using the statistical package Statistica 8.0 for Windows from StatSoft Inc. (Tulsa, OK, USA).

3. Results and discussion

3.1. Optimization procedure

The analysis of the effects using Pareto charts showed that the derivatization time and microwave power had significant effects on the derivatization of the amino acids (for a $p < 0.05$). The amount of the derivatization reagent did not have a significant effect. In addition, an important interaction was observed between the derivatization time and the microwave power for the derivatization of the amino acids. For this reason, these two parameters were used in a Doehlert design, and the amount derivatization reagent was set to 50.0 μL. The experimental conditions for the Doehlert

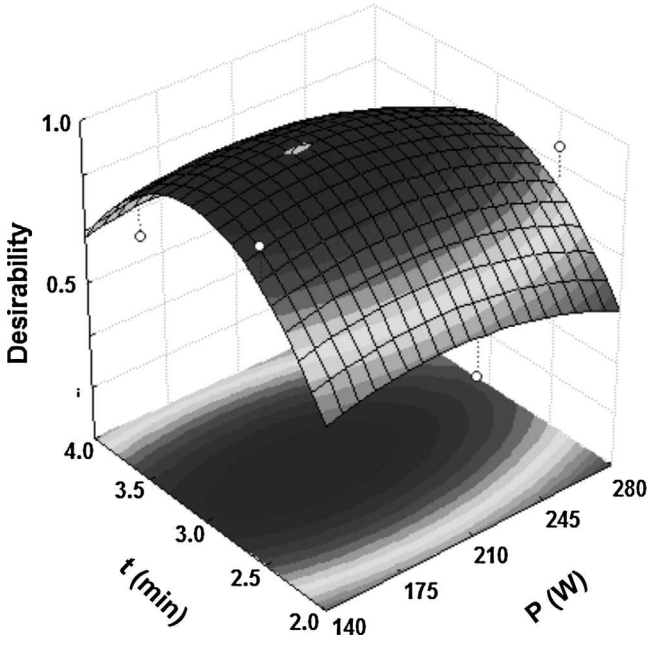

Fig. 1. Response surface for the combined desirability obtained from the Doehlert design experiments for all L-amino acids.

Table 3
Comparison with published methods.

Reference	Derivatization (time)	Method	LOQ (μmol L^{-1})	Precision (reproducibility)	Acuracy (recovery)	Number of amenable analytes	Matrices	Runtime
Schwarz et al. [13]	OPA-3MPA[1]	HPLC/DAD	12(citrulline) 423(glutamate)	1.6–5.9%	90–110%	23	Plasma	130 min total time analysis
Kaspar et al. [14]	iTRAQ[2] (30 min)	LC–MS/MS	0.5–50	≤18.34%	91–106%	20 L-AA	Urine	25 min
Kaspar et al. [14]	PCF[3] (30 min)	GC/MS	0.3–30	≤21.18%	98–111%	18 L-AA	Urine	20 min
Waldhier et al. [22]	MeOH/MCF[4]	GC–MS Rt-γDEXsa column	0.07–31.25	≤5%	87.2–119.3%	10 L-AA	Plasma and urine	42 min
Waterval et al. [11]	Underivatised	UPLC-MS/MS	40–287	6–10%	90–100%	22 L-AA	Plasma and urine	30 min
Visser et al. [17]	*s*-(NIFE)[5] (20 min)	UPLC-MS/MS	No report	3–18% CSF	85.5–102.6%	19 D-AA	CSF, Plasma and urine	25 min
Present study	BSTFA[6] (3 min)	GC/MS	0.02–7.07	6.4–18.7%	88–129%	16 L-AA	CSF	21 min

1 – o-phthalaldehyde 3-mercaptopropionic acid. 2 – isobaric tags for relative and absolute quantitation. 3 – propyl chloroformate. 4 – methanol/ethyl chloroformate. 5 – sodium tetraborate solution in acetonitrile and hydrochloric acid. 6 – N,O-bis-(trimethylsilyl) trifluoroacetamide.

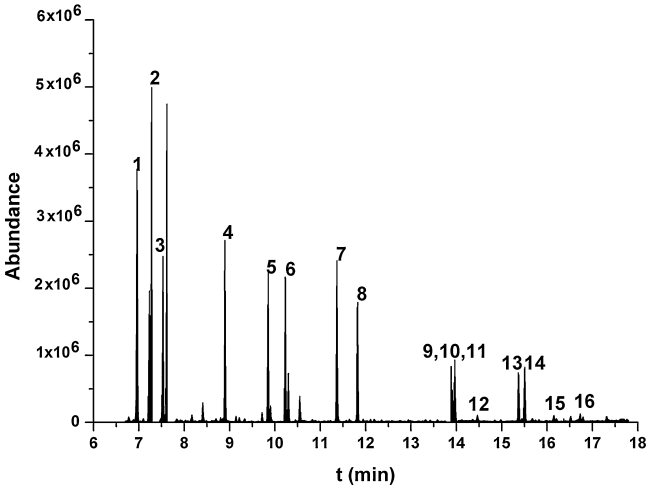

Fig. 2. Chromatographic profiles of the L-amino acid mixture (10 μmol L^{-1}) in aCSF. The component numbering is given in Table 1.

design for the real and coded values of each factor are shown in Table 2. To assess the effect of each variable on the response, the variables were coded to eliminate the influence of their different magnitudes. The central point was obtained in triplicate and was used to estimate the experimental error. An estimation of the coefficients ($b_0 - b_5$) of the second-degree polynomial model indicated by equation 1 was performed using the experimental responses. The level of significance for each regression was calculated by ANOVA and showed that the models fit well. The predictive ability under the experimental conditions used was assessed by the fractions of the explained variations (R^2), which ranged from 0.965 to 0.982. The response surfaces obtained from the regression models of each analyte were then combined using the desirability function, in which the geometric mean of the responses was computed. The desirability function can have a value ranging from 0 (undesirable) to 1 (desirable). Fig. 1 shows the desirability profile combined for all the amino acids according to the derivatization time and microwave power. The desirable conditions for the derivatization of all amino acids studied were 3 min for the derivatization time and 210 W for the microwave power. Fig. 2 shows the chromatogram obtained using these conditions.

3.2. Quality control and quality assurance

Linearity was established for all the analytes using CSF spiked with analytes in the concentration range of 0.1–133.0 μmol L^{-1}. Six concentrations were analyzed, with three measurements at each concentration. Because of the homoscedasticity of the instrumental responses, linear models for the calibration curves were constructed by the ordinary least squares method. The instrumental response was found to be linear in the range of concentrations studied, with coefficients of determination (R^2) above 0.990 ($p < 0.05$) for all amino acids except cysteine (0.975). The detection limits (LODs) and quantification limits (LOQs) were calculated from 10 measurements with 95% confidence according to the Eurachem Guidelines [31]. The amino acids had LODs between 0.01 μmol L^{-1} and 4.24 μmol L^{-1}, and the range of the LOQ was 0.02–7.07 μmol L^{-1}. The recovery test was performed with CSF samples spiked with 0 μmol L^{-1}, 0.1 μmol L^{-1}, 10.0 μmol L^{-1} and 100.0 μmol L^{-1} of each amino acid. The concentrations were calculated using a calibration curve prepared using the aCSF matrix. The accuracy was then calculated for each amino acid and ranged from 88.5 to 129%. Three identical aCSF matrices were spiked with 0.1 μmol L^{-1}, 10.0 μmol L^{-1} and 100.0 μmol L^{-1} of each amino acid to determine the intra-day and inter-day precision values. To

Table 4
Median L-amino acid concentrations and concentration ranges (μmol L^{-1}) in samples of human CSF (n = 16).

	Median	Range (μmol L^{-1})
L-Alanine	19.94	10.70–81.91
Glycine	6.88	5.59–14.41
Sarcosine	4.86	4.08–10.14
L-Valine	9.32	6.70–51.65
L-Leucine	6.70	5.02–29.59
L-Isoleucine	4.15	3.35–7.03
L-Serine	7.92	6.14–13.06
L-Threonine	7.58	4.52–10.55
L-Aspartic acid	10.43	9.91–20.53
L-Methionine	6.21	6.18–12.39
L-Proline	375	155.58–480.18
L-Cysteine	1046.72	550.27–2590.45
L-Glutamic acid	13.00	8.22–16.58
L-Phenylalanine	4.80	2.83–16.80
L-Asparagine	1.10	0.25–3.90
L-Lysine	10.37	9.74–20.47

determine the intra-day precision, the spiked CSF samples were derivatized independently and analyzed in one run. To determine the inter-day precision, spiked CSF samples were processed on three consecutive days. The precision was represented by the relative standard deviation (RSD). The intra-day precision ranged from 4.12 to 15.59%, and the inter-day precision ranged from 6.36 to 18.71%. These values are consistent with those obtained in other studies [21,26]. Table 1 lists the results of the method validation experiments. The Table 3 show a comparison of various published methods to analysis of amino acids in biological fluids. Our study presents advantages such as shorter time for derivatization (3 min), lower limits of quantification, below the expected levels of amino acids on biological fluids, and low cost per analysis. The optimized method also showed excellent reproducibility with RSD between 6.4 and 18.7% and the analytes studied are used to laboratorial diagnosis. However, amino acids such arginine and hystidine cannot be analyzed by GC/MS due to the thermal instability to the derivatives. Relative to recovery the range is larger that published methods, moreover, is acceptable to GC/MS methods.

3.3. Application of the method to samples of CSF

The validated MAD–GC/MS method for the determination of amino acid concentrations was used to analyze human CSF samples from 16 patients with AIDS. The results, reported in Table 4, indicate that this method had good performance with respect to the detection and quantification of amino acids when applied to real samples of human CSF. All 16 amino acids were detected, and the median concentrations of L-amino acids varied from 1.10 to 1046.72 μmol L^{-1}, with much higher concentrations observed for L-cysteine, L-proline, and L-alanine. L-Cysteine had the largest range, 550.27–2590.45 μmol L^{-1}, whereas L-asparagine had the smallest range, 0.25–3.90 μmol L^{-1}. The elevate levels of L-cysteine recorded in the present study could be the release of toxins since the patients analyzed presented neurological diseases. The values found for the other amino acids were consistent with those from other studies [12,26].

4. Conclusions

The results of this study indicate that microwave-assisted derivatization with BSTFA followed by GC/MS analysis is a suitable alternative technique for the determination of amino acid concentrations in human cerebrospinal fluid. An experimental design using a 2^3 two-level full factorial design (FFD), a Doehlert design and the desirability function were used to optimize the derivatization parameters. The derivatization is process is performed in a single step, limiting the use of reagents and resulting in a short analysis time. This approach has excellent linearity, good repeatability and reproducibility, and sufficiently low detection and quantification limits. The validated method was successfully applied to the analysis of L-amino acids in human CSF samples from patients with AIDS. Therefore, the proposed analytical protocol is a promising method that could be used in CSF and also to others biological fluids which includes urine, serum and tissues in the clinical diagnosis.

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Neurochemical study of amino acids in rodent brain structures using an improved gas chromatography–mass spectrometry method

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ABSTRACT

The analysis of amino acid levels is crucial for neuroscience studies because of the roles of these molecules as neurotransmitters and their influence on behavior. The present study describes the distribution and levels of 16 amino acids (alanine, asparagine, aspartic acid, cysteine, glycine, glutamic acid, isoleucine, leucine, lysine, methionine, phenylalanine, proline, sarcosine, serine, valine, and threonine) in brain tissues (prefrontal cortex, striatum, hippocampus and cerebellum) and the serum. Neurochemical analysis was performed on Wistar rats and C57BL/6 mice using an efficient method for extraction, a fast microwave-assisted derivatization and gas chromatography–mass spectrometry analysis. The amino acid concentration varied across brain regions for 14 of the 16 analyzed molecules, with detection limits ranging from $0.02 \pm 0.005\ \mu mol\ L^{-1}$ to $7.07 \pm 0.05\ \mu mol\ L^{-1}$. In rats, the concentrations of alanine, glycine, methionine, serine and threonine were higher in prefrontal cortex than in other areas, whereas in mice, the concentrations of glutamic acid, leucine and proline were highest in the hippocampus. In conclusion, this study provides a cerebral profile of amino acids in brain regions and the serum of rats and mice.

1. Introduction

The analysis of amino acid levels is crucial for the study of neuroscience because amino acids play important roles as neurotransmitters and can influence behavior under physiological conditions. In neuroscience, the amino acids are divided into non-neurotransmitters, which are important for structural and metabolic functions, and neurotransmitters, which are able to deliver messages across synapses (Shah et al., 2002). Amino acid neurotransmitters provide both excitatory and inhibitory neurotransmission in the central nervous system (CNS). The inhibitory amino acids (IAAs), including alanine, glycine, GABA, and taurine, reduce the activity of post-synaptic neurons. In contrast, the excitatory amino acids (EAAs), including aspartate, cysteine, homocysteine, and glutamate, increase the excitability of post-synaptic neurons (Shah et al., 2002).

Changes in amino acid levels in the CNS are widely associated with psychiatric and neurological disorders. EAAs can damage neurons by excessive stimulation; this phenomenon is called excitotoxicity and is involved in brain ischemia, epilepsy and Alzheimer's disease. Disruptions in glutamate neurotransmission have also been associated with schizophrenia and bipolar disorder. In addition, changes in neurotransmission by IAAs such GABA and glycine are associated with anxiety and some motor disorders (Bowery and Smart, 2006; Rajendra et al., 1997).

Abbreviations: aCSF, artificial cerebrospinal fluid; BSTFA, N,O-bis(trimethylsilyl)-trifluoroacetamide; CNS, central nervous system; EAA, excitatory amino acids; GC, gas chromatography; HPLC, high-performance liquid chromatography; IAA, inhibitory amino acids; LOD, detection limits; LOQ, quantification limits; MS, mass spectrometry; OPA, *o*-phthalaldehyde; MTBSTFA, N-(tert-butyldimethylsilyl)-N-methyltrifluoro-acetamide; TMCS, trimethylchlorosilane.

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The amino acid neurotransmitters can be found at concentrations ranging from nanomolar to micromolar in the CNS, requiring high-performance methods for accurate quantification of these molecules. These methods involve two steps. First, it is necessary to separate the neurotransmitters, which can be done by chromatography. Next, the molecules are detected, which depends on their chemical features. Amino acids are small aliphatic molecules and do not possess chemical features that permit direct analysis, as fluorescent emission or UV absorbance, necessitating an additional stage of derivatization (Shah et al., 2002). For analysis of amino acids, *o*-phthalaldehyde (OPA) is the most common derivatization agent, generating derivatives with fluorescent and electroactive features after reaction with primary amines in the presence of thiol groups. The amino acids derivatized with OPA are usually separated using high-performance liquid chromatography (HPLC) and analyzed with fluorescent detectors. However, there is an alternative method, which uses gas chromatography (GC). In this method, the amino acids are converted into more volatile analytes to be separated by GC, which can be monitored using either electron capture or mass spectrometric (MS) detection (Shah et al., 2002). The GC–MS method for detection of amino acids provides greater selectivity and ability to distinguish analytes, and it is more accurate than HPLC for the measurement of amino acids (Shah et al., 2002).

Because amino acids act as neurotransmitters on different cerebral substrates and in several neuropathologies, it is important to have effective methods for evaluating the profiles of these molecules in health and disease conditions. Therefore, this work describes the application of a recently developed GC–MS method (Paiva et al., 2013) in prefrontal cortex, striatum, hippocampus, cerebellum and serum samples from Wistar rats and C57BL/6 mice.

2. Materials and methods

2.1. Animals

Wistar rats (250–300 g) and C57BL/6 mice (25–30 g) of both sexes were used in this study. The animals were housed in plastic cages, maintained on a 12:12 h light–dark cycle and fed ad libitum. The experimental protocol was approved by the Ethics Committee for Animal Experimentation of our institution (Protocols N°. 042/11 and 182/13).

2.2. Chemicals and solutions

The artificial cerebrospinal fluid (aCSF) was manufactured with chemicals from Sigma–Aldrich (St. Louis, MO, USA) as follows: 127 mmol L^{-1} NaCl, 2 mmol L^{-1} KCl, 1.2 mmol L^{-1} KH_2PO_4, 26 mmol L^{-1} $NaHCO_3$, 2 mmol L^{-1} $MgSO_4$, 2 mmol L^{-1} $CaCl_2$, 10 mmol L^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 10 mmol L^{-1} glucose, bubbled with carbogenic mixture (95%, v/v O_2 and 5%, v/v CO_2).

The lysis buffer solution was prepared with a Sigma-FAST Protease Inhibitor cocktail Tablet (St. Louis, MO, USA), which contains 2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 mM Phosphoramidon, 130 mM Bestatin, 14 mM E-64, 1.0 mM Leupeptin, 0.2 mM Aprotinin, 10 mM Pepstatin A, and 50 mM sodium fluoride, and 1 mM sodium orthovanadate was added. The final pH was 7.20.

The following amino acid standards were obtained from Sigma–Aldrich (St. Louis, MO, USA): L-alanine, L-asparagine, L-aspartic acid, L-cysteine, L-glycine, L-glutamic acid, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, sarcosine, L-serine, L-valine, L-threonine. The standard solution of each amino acid was prepared at an initial concentration of 1.0 mg mL^{-1} in H_2O and stored at −20 °C. Primary standard mixtures, from the separate standard solutions, containing 10 μg mL^{-1} of the indicated amino acids, were prepared in H_2O. These working solutions were stored at 4 °C.

HPLC-grade methanol was obtained from Merck KGaA (Darmstadt, Germany), *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS) 99:1 (v/v), and pyridine anhydrous 99.8% (m/m) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and methoxyamine hydrochloride 98% m/m was obtained from Fluka (St. Louis, MO, USA).

2.3. Collecting and sample preparation procedures

The animals were euthanized by decapitation, and blood samples were collected. Next, the brain was rapidly removed from the cranium and submerged in ice-cold aCSF. The regions of interest (prefrontal cortex, striatum, hippocampus and cerebellum) were dissected out on an ice-cold plate as described by Chiu et al. (2007). Then, each component was weighed, placed in a 1.5 mL microcentrifuge tube and homogenized in 400 μL of lysis buffer solution for 30 s. After homogenization, the samples were centrifuged (8000 × *g* for 10 min at 4 °C), and aliquots of the supernatants were stored at −20 °C until the derivatization step. The blood samples were centrifuged to collect the serum (2000 × *g* for 5 min at 4 °C).

2.4. Derivatization procedure

The derivatization procedure was performed as described in Paiva et al. (2013). Briefly, an aliquot of 100 μL of supernatants homogenate was transferred to a tube (or 50.0 μL, for cerebellum and serum), and to precipitate the protein was added 900 μL of methanol at −10 °C for each tube. The solution was vortexed for 1 min and centrifuged at 10,000 rpm for 10 min at room temperature. Then, 100 μL of supernatant was transferred to a glass GC vial and evaporated to dryness at room temperature under N_2. Next, 15 μL of methoxyamine in pyridine at 20 mg mL^{-1} was added, followed by 35 μL of BSTFA with 1% TMCS. The solution was vortexed for 30 s and submitted to microwave irradiation in a domestic microwave oven (700 W power) equipped with a turning table for 3 min. The final solution was used for GC–MS analysis.

2.5. Analysis of gas chromatography–mass spectrometry

The amino acid analyses were performed using a Shimadzu (Kyoto, Japan) GC–MS system model GC-2010/QP-2010 high performance quadrupole. The mass spectrometer operated in electron impact mode (EI) at 70 eV. The analytes were separated on a Restek (Bellefont, PA, USA) Rtx-5MS fused-silica capillary column chemically bonded (30 m × 0.25 mm id × 0.25 μm film thickness) containing 5% diphenyl, 95% dimethylpolysiloxane. The oven temperature program began at 80 °C, rose to 200 °C at 8 °C min^{-1}, rose to 300 °C at 30 °C min^{-1}, and held that temperature for 3 min. The injector was operated at 280 °C in splitless mode for 3 min, followed by a 1:20 split ratio. Helium with a purity of a 99.99% was used as the carrier gas, at a flow rate of 1.0 mL min^{-1}. Samples were injected manually with a 10 μL microsyringe Hamilton (Reno, NV, USA), in splitless mode, with a 1 min vent open time, and an injected volume of 1.0 μL. The ion source temperature was 200 °C and the GC–MS interface was kept on 260 °C. For identification and confirmation, each amino acid solution was prepared separately for derivatization in optimized conditions. Next, 1.0 μL of solution was injected into the GC–MS apparatus, and the analysis was performed in full scan mode (range 45–300 *m*/*z*), with a scan time of 2 scan s^{-1}. The retention times achieved and ions used for

identification and quantification are shown in Fig. 1. The quantification was achieved in single ion monitoring. Signal acquisition and data processing were performed using the Labsolution software Shimadzu (Kyoto, Japan). A domestic microwave from Philco (model PRC-700W, São Paulo, Brazil) was used for microwave-assisted derivatization.

The method parameters of merit were performed according to (EURACHEM, 2002) guidelines. The lysis buffer solution was used as a blank matrix. Six concentration levels were analyzed, with three measurements at each concentration level. The linear models for the calibration curves were constructed by the standard least squares method. Linearity was established for all the analytes from 0.02 to 100.00 μmol L^{-1}.

2.6. Statistics

Group means were compared via two-way ANOVA followed by Bonferroni's post-test. The results were expressed as the mean ± SEM. Statistical significance was established at $P < 0.05$. In addition, factor analysis was applied, using principal component analysis for factor extraction, with varimax normalized rotation for factor loading and marked loadings greater than 0.70.

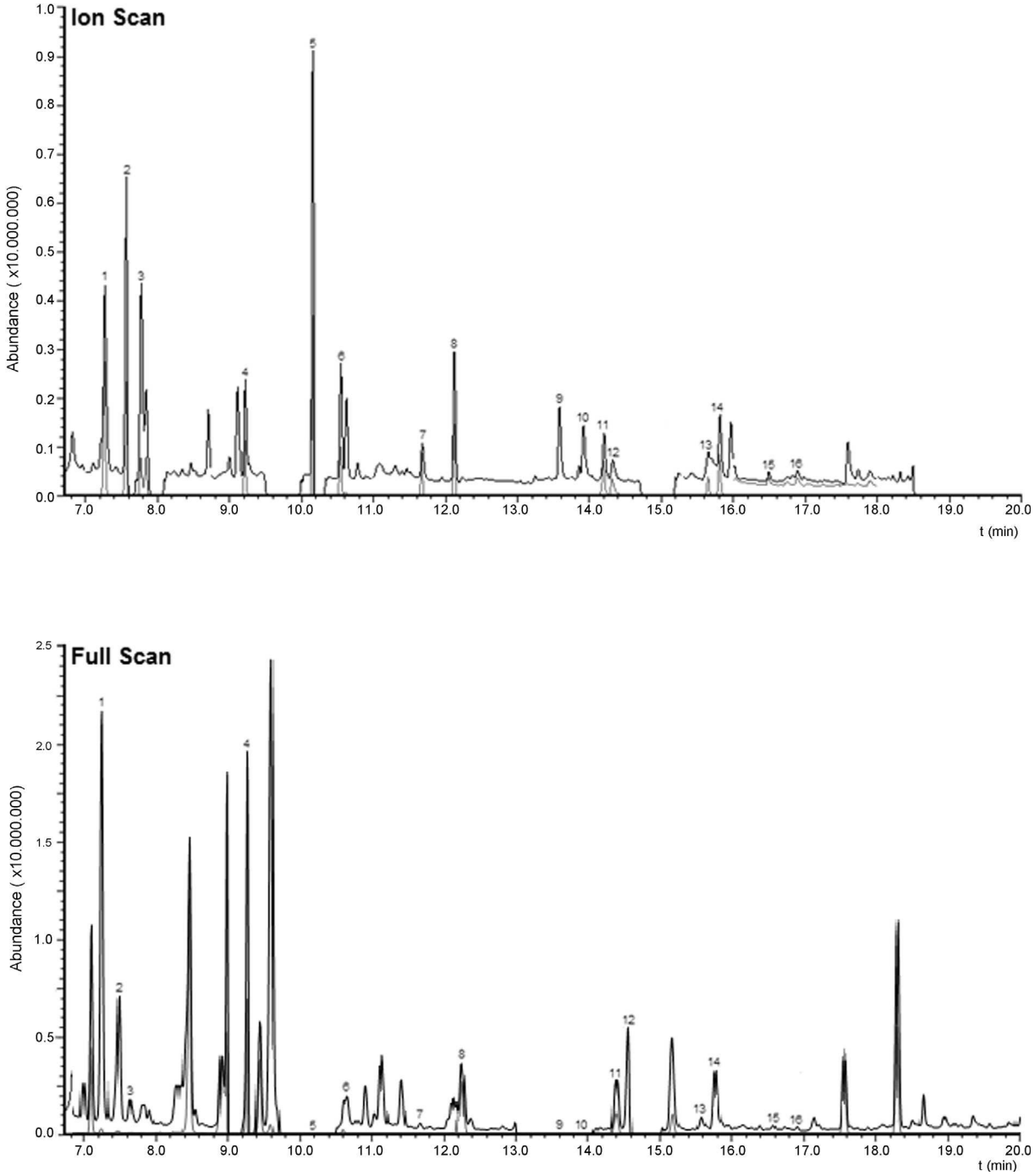

Fig. 1. Representative chromatogram of standard amino acids. The output order was L-alanine (1), glycine (2), sarcosine (3), L-valine (4), L-leucine (5), L-isoleucine (6), L-serine (7), L-threonine (8), L-aspartic acid (9), L-methionine (10), L-proline (11), L-cysteine (12), L-glutamic acid (13), L-phenylalanine (14), L-asparagine (15) and L-lysine (16).

Table 1
Amino acid levels in the brain and serum from Wistar rats.

Amino acids	Prefrontal cortex		Striatum		Hippocampus		Cerebellum		Serum	
(μg/mg of proteins)	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Alanine	6.81 ± 1.11	14.07 ± 2.24	5.36 ± 0.56	7.23 ± 0.84	8.35 ± 0.54	9.22 ± 0.59	7.41 ± 0.78	6.17 ± 1.72	13.05 ± 1.40	2.46 ± 1.60
Glycine	3.17 ± 0.41	4.91 ± 1.10	1.13 ± 0.13	1.73 ± 0.23	3.99 ± 1.86	8.15 ± 1.46	2.24 ± 0.28	2.62 ± 0.28	0.91 ± 0.03	0.89 ± 0.05
Sarcosine	1.51 ± 0.11	1.67 ± 0.14	5.77 ± 3.76	1.35 ± 0.13	1.17 ± 0.33	0.93 ± 0.05	5.11 ± 3.40	5.94 ± 4.17	4.96 ± 4.31	2.67 ± 2.00
Valine	1.48 ± 0.10	2.41 ± 0.26	1.00 ± 0.05	2.63 ± 1.42	1.15 ± 0.11	1.21 ± 0.07	1.69 ± 0.20	1.26 ± 0.15	6.63 ± 0.80	1.20 ± 0.66
Leucine	11.05 ± 2.48	19.65 ± 4.26	12.72 ± 2.12	19.04 ± 1.85	12.88 ± 2.92	15.32 ± 1.07	10.40 ± 1.10	10.18 ± 3.56	5.23 ± 0.67	1.20 ± 0.65
Isoleucine	1.34 ± 0.08	1.58 ± 0.22	0.73 ± 0.07	1.10 ± 0.09	0.69 ± 0.11	0.64 ± 0.03	1.31 ± 0.18	1.39 ± 0.08	2.72 ± 0.34	0.44 ± 0.01
Serine	6.79 ± 1.10	17.35 ± 2.98	3.35 ± 0.47	5.99 ± 0.70	7.07 ± 0.59	9.21 ± 1.25	5.35 ± 1.01	5.15 ± 1.18	6.27 ± 0.90	0.82 ± 0.01
Threonine	3.38 ± 0.51	7.59 ± 1.26	2.02 ± 0.20	3.71 ± 0.43	3.23 ± 0.34	3.88 ± 0.53	3.48 ± 0.13	3.74 ± 1.03	5.34 ± 0.83	0.44 ± 0.01
Aspartic acid	30.08 ± 8.05	35.75 ± 9.17	24.86 ± 5.07	36.70 ± 4.78	24.76 ± 4.75	29.33 ± 3.58	26.20 ± 4.75	28.62 ± 10.30	1.85 ± 0.20	1.28 ± 0.02
Methionine	2.14 ± 0.20	2.39 ± 0.19	1.27 ± 0.14	1.86 ± 0.17	1.23 ± 0.15	1.26 ± 0.07	2.36 ± 0.30	2.43 ± 0.07	1.36 ± 0.23	0.84 ± 0.01
Proline	171.2 ± 54.24	119.93 ± 64.62	127.36 ± 27.75	180.25 ± 31.13	108.49 ± 42.50	186.93 ± 11.19	0.89 ± 0.11	199.56 ± 81.67	32.92 ± 9.53	0.28 ± 0.02
Cysteine	36.17 ± 8.92	92.25 ± 15.59	27.51 ± 1.61	42.32 ± 4.18	205.85 ± 109.40	112.89 ± 25.22	19.80 ± 7.02	28.72 ± 12.03	31.25 ± 6.92	1.46 ± 0.74
Glutamic Acid	93.90 ± 26.82	104.58 ± 42.36	43.19 ± 8.79	105.89 ± 11.47	80.37 ± 26.99	86.46 ± 15.12	14. 18 ± 1.80	84.07 ± 34.42	2.76 ± 0.92	1.14 ± 0.02
Phenylalanine	1.05 ± 0.07	1.50 ± 0.53	1.06 ± 0.14	2.30 ± 0.21	0.70 ± 0.08	0.80 ± 0.21	1.20 ± 0.14	1.13 ± 0.06	1.11 ± 0.36	0.39 ± 0.01
Aparagine	0.29 ± 0.10	0.35 ± 0.20	0.38 ± 0.06	0.48 ± 0.06	0.41 ± 0.11	0.63 ± 0.18	0.39 ± 0.07	0.59 ± 0.21	0.51 ± 0.23	0.04 ± 0.02
Lysine	3.15 ± 0.35	3.27 ± 0.27	1.89 ± 0.23	2.57 ± 0.24	1.70 ± 0.22	1.82 ± 0.11	3.66 ± 0.53	4.89 ± 1.24	1.36 ± 0.06	1.34 ± 0.01

Mean ± SEM.

3. Results

3.1. Amino acid extraction and gas chromatography–mass spectrometry

The extraction with lysis buffer solution and GC–MS analysis showed good linearity in the range studied; the curves obtained R^2 = 0.975 (L-cysteine) to 0.998 (L-aspartic acid). Detection limits (LOD) and quantification limits (LOQ) were calculated from the mean and standard deviation of ten blank measurements with 95% confidence. The LOD ranged from 0.01 ± 0.005 μmol L^{-1} to 4.23 ± 0.02 μmol L^{-1} for L-glutamic acid and L-lysine, respectively. The LOQ ranged from 0.02 ± 0.005 μmol L^{-1} to 7.07 ± 0.05 μmol L^{-1} for the same analytes. In assessing the intra-assay precision (repeatability) and inter-day precision (reproducibility), ten replicates at the three concentration levels were analyzed the same day and on consecutive days, respectively. The coefficient of variation for repeatability was 4.12–15.59%. The coefficient of variation for inter-day precision ranged from 6.36% to 17.74%. Recovery study was performed with spiked free amino acids in the lysis buffer solution. The recovery ranged from 88.50% (L-lysine) to 129.40% (L-glutamic acid).

3.2. Amino acid content in Wistar rat brain tissue and serum

The amino acid content in Wistar rats was evaluated in four brain regions (prefrontal cortex, striatum, hippocampus and cerebellum) and in the serum by GC–MS (Table 1). To improve the understanding of these results, the amino acids were grouped by chemical features, and differences between tissues and genders were analyzed. Fig. 2 shows representative chromatograms from brain regions and serum of Wistar rats.

The first group of amino acids analyzed was aliphatic amino acids, which includes alanine, glycine, sarcosine, leucine and isoleucine (Fig. 3). Alanine content was higher in the cortex than in the striatum ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test). A similar difference was observed in glycine content, where the cortex level was higher than that found in striatum and serum ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test). Furthermore, the hippocampal level of glycine was higher than that found in striatum, cerebellum and serum ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test). Leucine levels did not differ among the brain tissues studied ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test), but it was lower in serum than in prefrontal cortex, striatum or hippocampus ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test). Isoleucine levels were higher in prefrontal cortex than in hippocampus and striatum, the hippocampal isoleucine level was lower than the cerebellar. In contrast with other members of this group, the valine level was higher in serum than in brain tissues. There was no difference in the amount of sarcosine between the analyzed tissues ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test).

The second group analyzed was the hydroxyl amino acids, serine and threonine (Fig. 3). Serine levels were higher in the cortex than in the other tissues, and the hippocampal level was significantly higher than the serum level ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test). The threonine level in cortex was higher than in striatum and serum ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test).

The third group was the sulfur-containing amino acids (Fig. 4). The amounts of methionine in the cortex and cerebellum were higher than the levels in striatum, hippocampus and serum ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test). In contrast, the amount of cysteine in the hippocampus was higher than that in the striatum or in serum ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test).

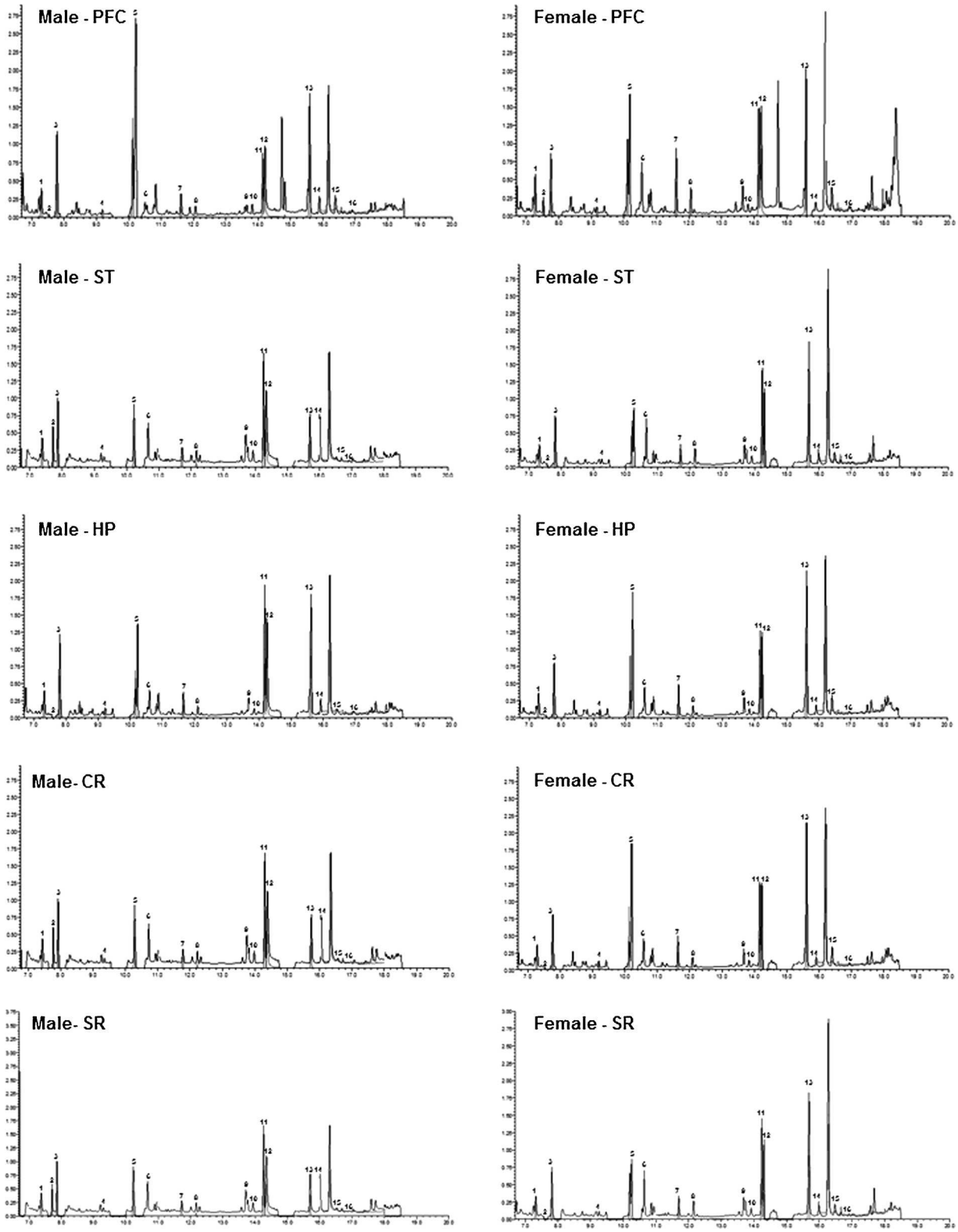

Fig. 2. Representative chromatograms of brain regions and serum of male and female Wistar rats. The prefrontal cortex (PFC), striatum (ST), hippocampus (HP), cerebellum (CR) and serum (SR) were analyzed as described in Section 2.

The fourth group analyzed was the acidic amino acids (glutamic acid, aspartic acid and its amide asparagine) (Fig. 4). The levels of glutamate and aspartate were higher in brain tissue than in the serum, with no significant differences between the brain tissues ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test). The amide asparagine did not show differences between the analyzed tissues ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test). The other three groups, cyclic amino acid (proline), aromatic amino acid (phenylalanine) and basic amino acid (lysine), were also analyzed. The amount of proline was higher in striatum than in serum ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test). The amount of phenylalanine was higher in the striatum than

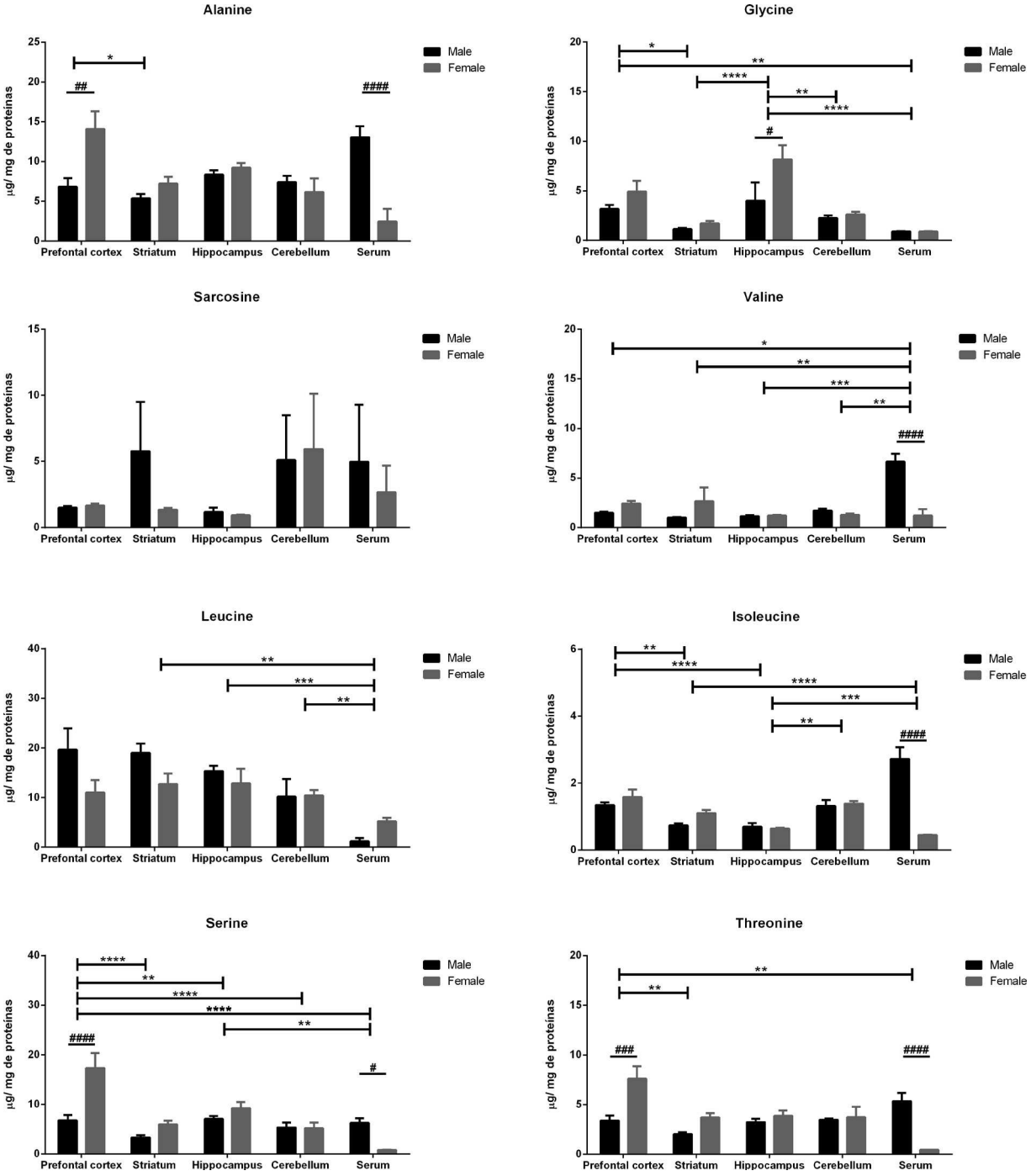

Fig. 3. Brain content of amino acids in Wistar rats. The amino acids were determined using gas chromatography–mass spectrometry. Four brain structures (prefrontal cortex, striatum, hippocampus and cerebellum) and serum were analyzed. The results are expressed as the mean ± SEM from at least five different animals ($P > 0.05$, two-way ANOVA, with Bonferroni's post hoc test).

in the hippocampus. Lysine levels were higher in the cerebellum than in the other brain tissues and greater in the striatum than in the serum ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test).

In Wistar rats, most of the gender differences were found in serum, where alanine, valine, threonine, isoleucine and serine were significantly higher in male than in female samples ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test). In prefrontal cortex, alanine and threonine were higher in females than males, whereas serine was higher in males than females. Glycine and phenylalanine were significantly higher in females in the hippocampus and striatum, respectively ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test).

Based on the results generated by the data matrix analyses of amino acids in different brain regions of Wistar rats, we decided to perform an exploratory factorial analysis to understand the distribution of these molecules in the brain (Fig. 5). In the prefrontal cortex, the amino acids A, T, S, V, L and C are associated with factor 1, whereas the amino acids Sr, M, K, D and E are associated with factor 2. In hippocampus, the amino acids A, T, S, G, V, P, C, D and E are associated with factor 1 and the amino acids Sr, L, I, F, M and K with factor 2. In cerebellum, factor 1 is composed of

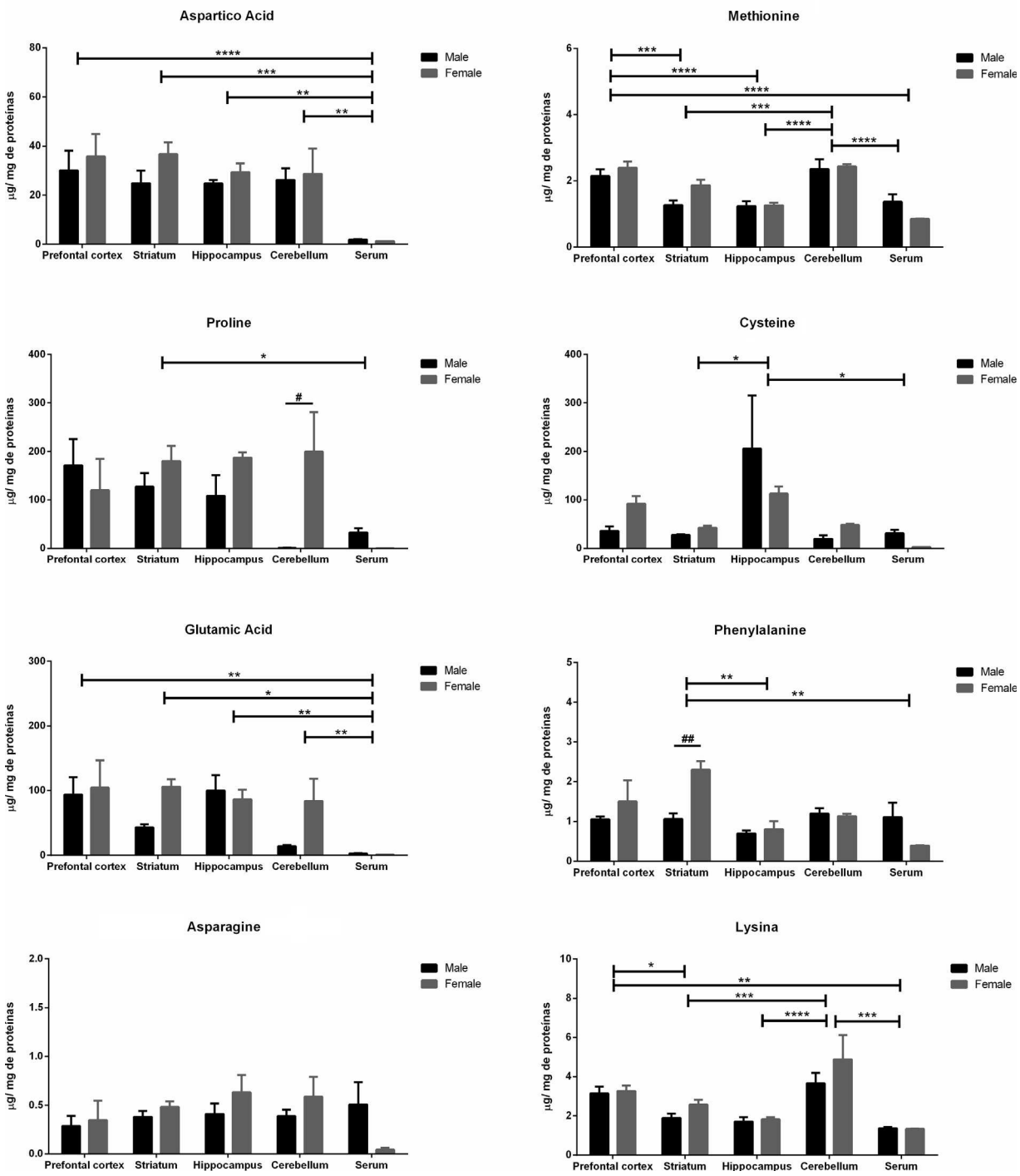

Fig. 4. Brain content of amino acids in Wistar rats. The amino acid levels were determined using gas chromatography–mass spectrometry. Four brain structures (prefrontal cortex, striatum, hippocampus and cerebellum) and serum were analyzed. The results are expressed as the mean ± SEM from at least five different animals ($P > 0.05$, two-way ANOVA, with Bonferroni's post hoc test).

the amino acids A, T, S, L, P, C, D and E and factor 2 of the amino acids G, V, I, M and F. In the striatum, the amino acids A, T, S, F, C, D and E are associated with factor 1, and the amino acids G, V, L, I, M and K with factor 2.

3.3. Amino acid contents in C57BL/6 mouse brain tissue and serum

The amino acid content was evaluated in four cerebral regions (prefrontal cortex, striatum, hippocampus and cerebellum) and in the serum by GC–MS from C57BL/6 mice (Table 2). The chromatogram profile is shown in Fig. 6.

The aliphatic amino acids (alanine, glycine, sarcosine, leucine and isoleucine) were also analyzed in mice (Fig. 7). Alanine content was higher in the cortex when compared with striatum and serum and was also higher in hippocampus than in striatum ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test). Glycine content was also higher in the cortex than in striatum and serum, higher in the hippocampus than in the striatum and serum, and higher in the cerebellum than in the hippocampus and serum ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test). Sarcosine content was higher in cerebellum than in prefrontal cortex, hippocampus and serum. Sarcosine was also more concentrated in the cortex

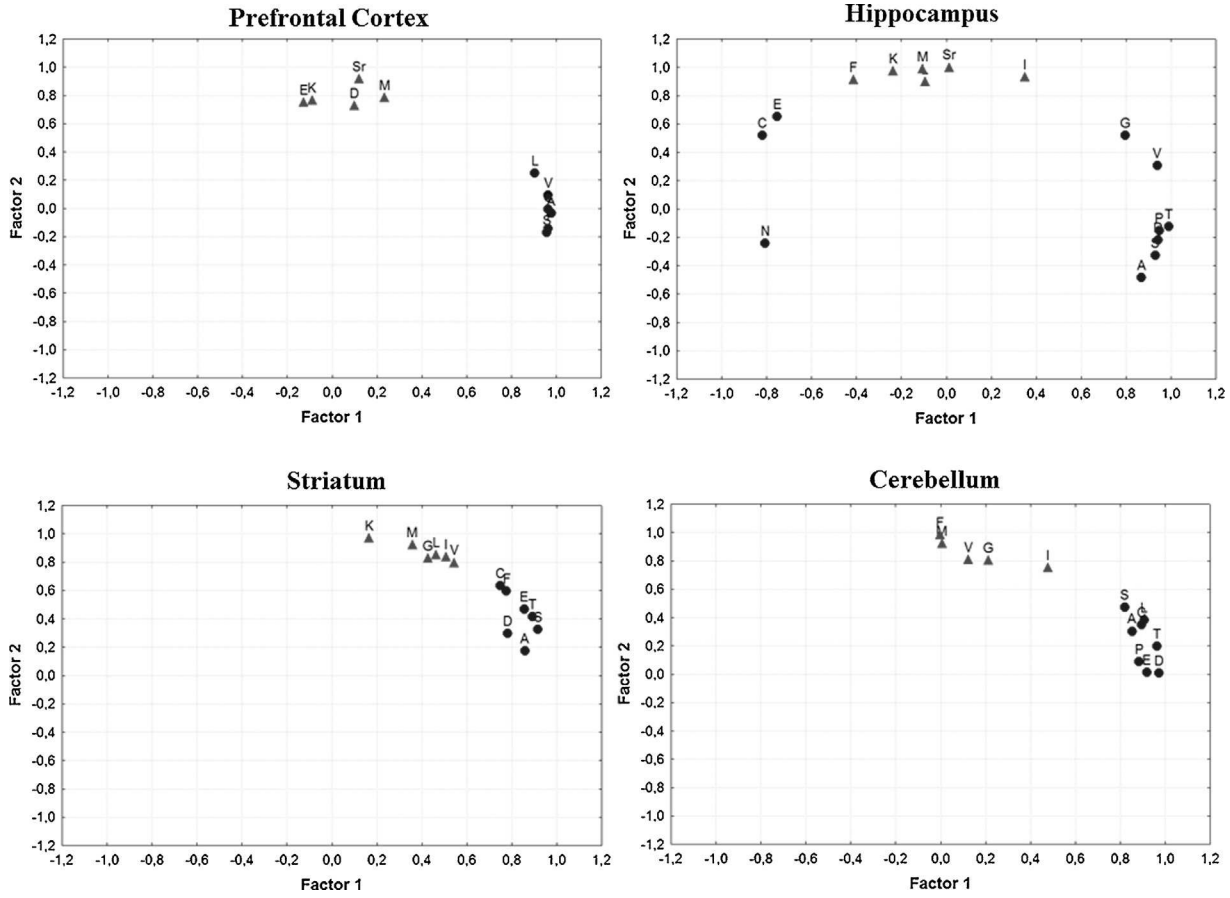

Fig. 5. Factorial analysis of brain levels of amino acids in Wistar rats. Alanine (A), glycine (G), sarcosine (Sr), L-valine (V), L-leucine (L), L-isoleucine (I), L-serine (S), L-threonine (T), L-aspartic acid (D), L-methionine (M), L-proline (P), L-cysteine (C), L-glutamic acid (E), L-phenylalanine (F), L-asparagine (N) and L-lysine (K). The data were extracted by the principal components method; varimax normalization was used as factor Loadings and was marked for loadings greater than 0.70.

than in the striatum and serum. The valine content was lower in striatum than in prefrontal cortex and cerebellum ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test). Leucine content was higher in hippocampus than in prefrontal cortex, cerebellum and serum ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test), whereas isoleucine showed no differences among the brain tissues studied ($P > 0.05$, two-way ANOVA, with Bonferroni's post hoc test).

The hydroxyl amino acids (serine and threonine) were also analyzed (Fig. 7). Serine content was higher in the cortex than in the striatum and serum, lower in striatum than hippocampus and cerebellum, and more concentrated in cerebellum than serum ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test). In contrast, threonine levels showed no differences among the brain tissues studied ($P > 0.05$, two-way ANOVA, with Bonferroni's post hoc test).

Table 2
Amino acid levels in the brain and serum from C57BL/6 mice.

Amino acids	Prefrontal cortex		Striatum		Hippocampus		Cerebellum		Serum	
(μg/mg of proteins)	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Alanine	7.05 ± 0.88	6.27 ± 0.76	3.32 ± 0.39	3.48 ± 0.50	4.32 ± 1.09	8.04 ± 1.46	6.31 ± 0.46	5.08 ± 0.62	10.34 ± 5.81	2.99 ± 0.78
Glycine	4.98 ± 0.19	4.79 ± 0.23	2.41 ± 0.19	2.61 ± 0.16	3.05 ± 0.34	4.78 ± 1.05	5.36 ± 0.33	5.49 ± 0.77	1.92 ± 0.10	2.13 ± 0.28
Sarcosine	3.32 ± 0.12	3.22 ± 0.18	5.29 ± 3.73	6.42 ± 4.76	13.48 ± 7.08	3.00 ± 0.59	4.48 ± 0.90	3.72 ± 0.54	1.31 ± 0.06	1.46 ± 0.19
Valine	2.28 ± 0.13	2.17 ± 0.10	1.11 ± 0.11	1.16 ± 0.08	1.48 ± 0.19	1.95 ± 0.30	2.35 ± 0.14	2.39 ± 0.32	6.01 ± 3.30	1.36 ± 0.33
Leucine	10.16 ± 0.82	9.69 ± 0.71	9.11 ± 1.11	12.70 ± 1.00	11.19 ± 4.07	25.07 ± 4.88	9.89 ± 1.20	6.95 ± 1.74	6.67 ± 4.39	1.45 ± 0.20
Isoleucine	2.42 ± 0.09	2.31 ± 0.12	2.74 ± 1.50	1.28 ± 0.07	1.49 ± 0.18	2.09 ± 0.34	2.49 ± 0.19	2.62 ± 0.38	2.65 ± 1.17	1.11 ± 0.14
Serine	4.83 ± 0.30	4.60 ± 0.24	2.48 ± 0.27	2.70 ± 0.22	3.24 ± 0.50	5.26 ± 0.79	4.84 ± 0.33	4.99 ± 0.70	1.93 ± 0.09	1.98 ± 0.25
Threonine	2.62 ± 0.12	2.49 ± 0.13	1.37 ± 0.13	1.49 ± 0.11	1.71 ± 0.25	2.68 ± 0.45	2.97 ± 0.13	2.83 ± 0.34	3.21 ± 1.56	1.31 ± 0.19
Aspartic Acid	17.49 ± 2.49	14.85 ± 3.42	15.89 ± 4.21	10.86 ± 3.09	9.68 ± 2.67	22.69 ± 5.78	36.53 ± 4.55	21.04 ± 6.94	4.80 ± 2.15	3.02 ± 0.42
Methionine	4.68 ± 0.17	4.48 ± 0.24	2.29 ± 0.20	2.48 ± 0.13	2.87 ± 0.34	4.02 ± 0.70	4.88 ± 0.37	5.12 ± 0.74	2.15 ± 0.41	1.98 ± 0.28
Proline	70.28 ± 9.86	69.86 ± 12.26	47.80 ± 7.86	50.34 ± 11.15	59.37 ± 15.98	132.30 ± 37.06	127.20 ± 13.91	55.38 ± 27.13	29.65 ± 18.49	4.28 ± 3.18
Cysteine	21.47 ± 0.89	20.05 ± 2.14	11.77 ± 1.59	13.19 ± 2.62	14.91 ± 2.89	26.79 ± 4.93	20.42 ± 0.66	15.39 ± 1.47	117.60 ± 70.14	176.70 ± 85.09
Glutamic Acid	7.95 ± 0.77	7.33 ± 1.30	6.06 ± 1.99	8.00 ± 3.52	12.50 ± 5.39	30.69 ± 11.65	13.91 ± 3.8	10.66 ± 2.66	6.39 ± 4.09	2.64 ± 0.37
Phenylalanine	2.13 ± 0.08	2.04 ± 0.10	1.06 ± 0.11	1.18 ± 0.08	1.42 ± 0.19	2.20 ± 0.41	2.28 ± 0.15	2.35 ± 0.32	1.67 ± 0.85	0.92 ± 0.12
Aparagine	0.30 ± 0.11	0.15 ± 0.02	0.11 ± 0.03	0.10 ± 0.01	0.10 ± 0.02	0.15 ± 0.02	0.15 ± 0.02	0.13 ± 0.02	0.12 ± 0.04	0.07 ± 0.02
Lysine	7.29 ± 0.26	6.94 ± 0.38	3.52 ± 0.27	3.84 ± 0.21	4.48 ± 0.54	6.16 ± 1.09	7.54 ± 0.58	7.95 ± 1.20	2.79 ± 0.10	3.11 ± 0.43

Mean ± SEM.

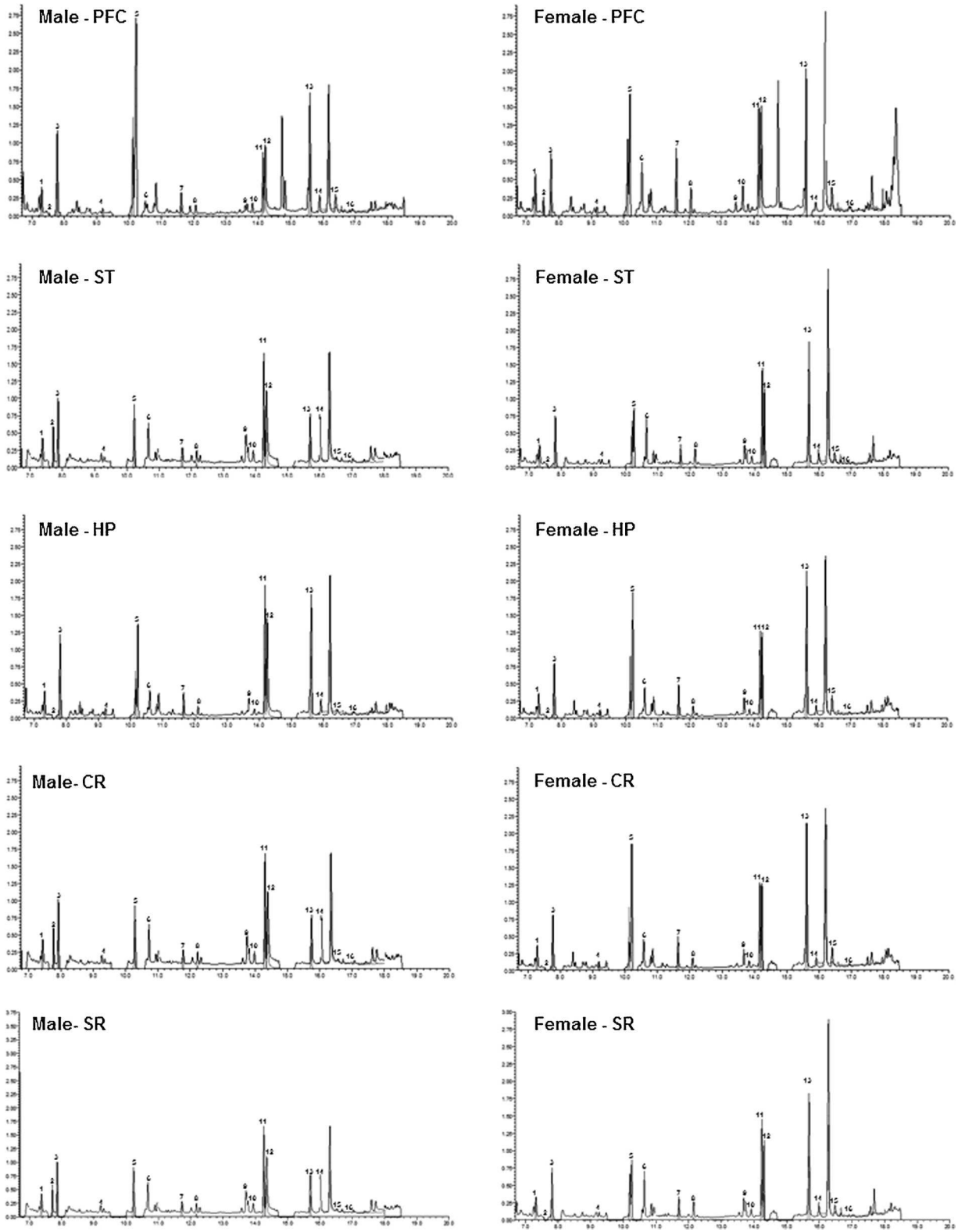

Fig. 6. Representative chromatograms of brain regions and serum of male and female C57BL/6 mice. The prefrontal cortex (PFC), striatum (ST), hippocampus (HP), cerebellum (CR) and serum (SR) were analyzed as described in Section 2.

The sulfur-containing amino acids (methionine and cysteine) were analyzed in mice (Fig. 8). Methionine content was higher in the cortex than in striatum and serum ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test). Furthermore, the cerebellar level of methionine was greater than that of the serum or hippocampus. Cysteine, the other sulfur-containing amino acid, was present at higher levels in blood than in other tissues ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test).

The acidic amino acids (glutamic acid, aspartic acid and its amide asparagine) were also evaluated (Fig. 8). The level of aspartic acid was higher in cerebellum than in other tissues ($P < 0.05$, Two-way ANOVA, with Bonferroni's post hoc test), whereas the level of

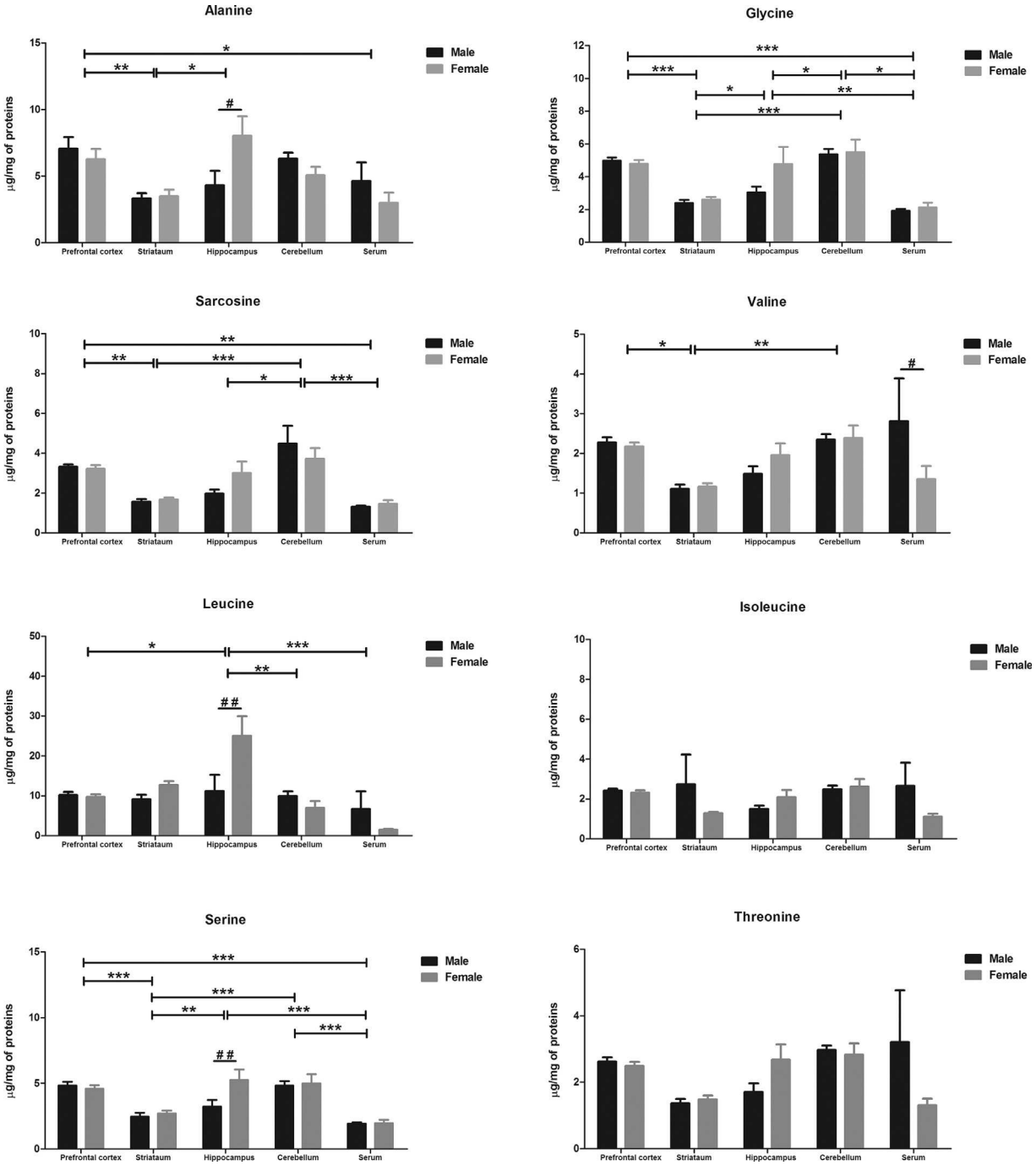

Fig. 7. Brain content of sulfur-containing amino acids in C57BL/6 mice. Levels of the amino acids methionine and cysteine were determined using gas chromatography–mass spectrometry. Four brain structures (prefrontal cortex, striatum, hippocampus and cerebellum) and serum were analyzed. The results are expressed as the mean ± SEM from at least five different animals ($P > 0.05$, two-way ANOVA, with Bonferroni's post hoc test).

glutamic acid was higher in hippocampus than in other tissues. Asparagine showed no differences between the analyzed tissues ($P > 0.05$, two-way ANOVA, with Bonferroni's post hoc test).

The levels of proline (cyclic amino acid), phenylalanine (aromatic amino acid) and lysine (basic amino acid) were also analyzed (Fig. 8). Proline content was lower in serum when compared with hippocampus and cerebellum ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test). The amount of phenylalanine was higher in cortex, hippocampus and cerebellum than in striatum and serum ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test). Lysine levels were higher in cortex than striatum and serum, and lysine was also more concentrated in cerebellum than striatum, hippocampus and serum ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test).

In C57BL/6 mice, most of the gender differences were found in hippocampus, where alanine, leucine, serine and proline were higher in females than males ($P < 0.05$, two-way ANOVA, with Bonferroni's post hoc test). In cerebellum, aspartic acid and proline levels were higher in males than females, whereas valine levels were higher in male mice.

The exploratory factor analysis revealed that in prefrontal cortex, the amino acids G, V, I, S, T, F, K are associated with factor 1 and the amino acids A, C, P, D, E with factor 2 (Fig. 9). In hippocampus, we find that amino acids G, V, I, M, C, N, F, K are

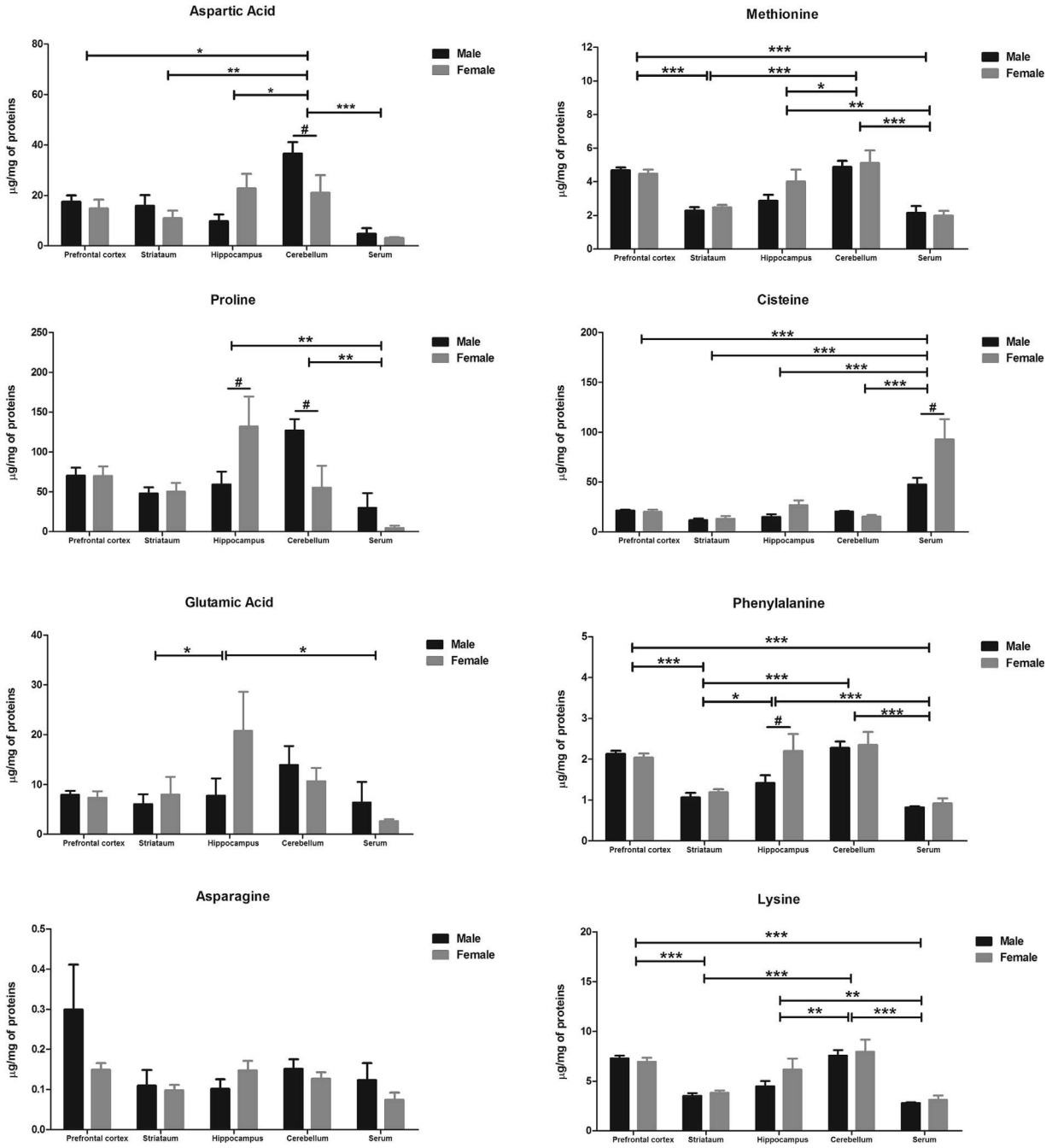

Fig. 8. Brain content of acidic amino acids in C57BL/6 mice. The levels of acidic amino acids (glutamate, aspartate, and asparagine) were determined using gas chromatography–mass spectrometry. Four brain structures (prefrontal cortex, striatum, hippocampus and cerebellum) and serum were analyzed. The results are expressed as the mean ± SEM from at least five different animals ($P > 0.05$, two-way ANOVA, with Bonferroni's post hoc test).

associated with factor 1, and amino acids A, L, S, P, D and E with factor 2. In cerebellum, the amino acids A, G, V, I, S, T, M, F and K are associated with factor 1, and the amino acids L, C, P, D and E are associated with factor 2. The most distinctive pattern was found in the striatum, where factor 1 is composed of the amino acids A, G, V, S, T, M, F, K, C, P, D and E, and factor 2 is composed of the amino acids I, N.

4. Discussion

The measurement of amino acid levels from brain tissue using lysis buffer solution extraction, BSTFA microwave derivatization and CG–MS analysis showed great sensitivity and rapid performance. Amino acid analysis in brain tissues can be performed by several chromatography methods (HPLC with photodiode array and fluorescence detection, liquid chromatography–tandem mass spectrometry (LC–MS/MS) or ultra-high-performance-liquid-chromatography–tandem mass spectrometry (UPLC–MS/MS)); however, the most common method uses ion-exchange liquid chromatography followed by UV detection (Schwarz et al., 2005; Kaspar et al., 2009; Waldhier et al., 2010; Waterval et al., 2009; Visser et al., 2011). This method is useful, but it can be time-consuming, and the sensitivity is often low. On the other hand, GC/MS methods present good separation, high sensitivity, robustness and large spectrum libraries, and these technical advantages are responsible for good results in metabolite analysis (Shah et al., 2002).

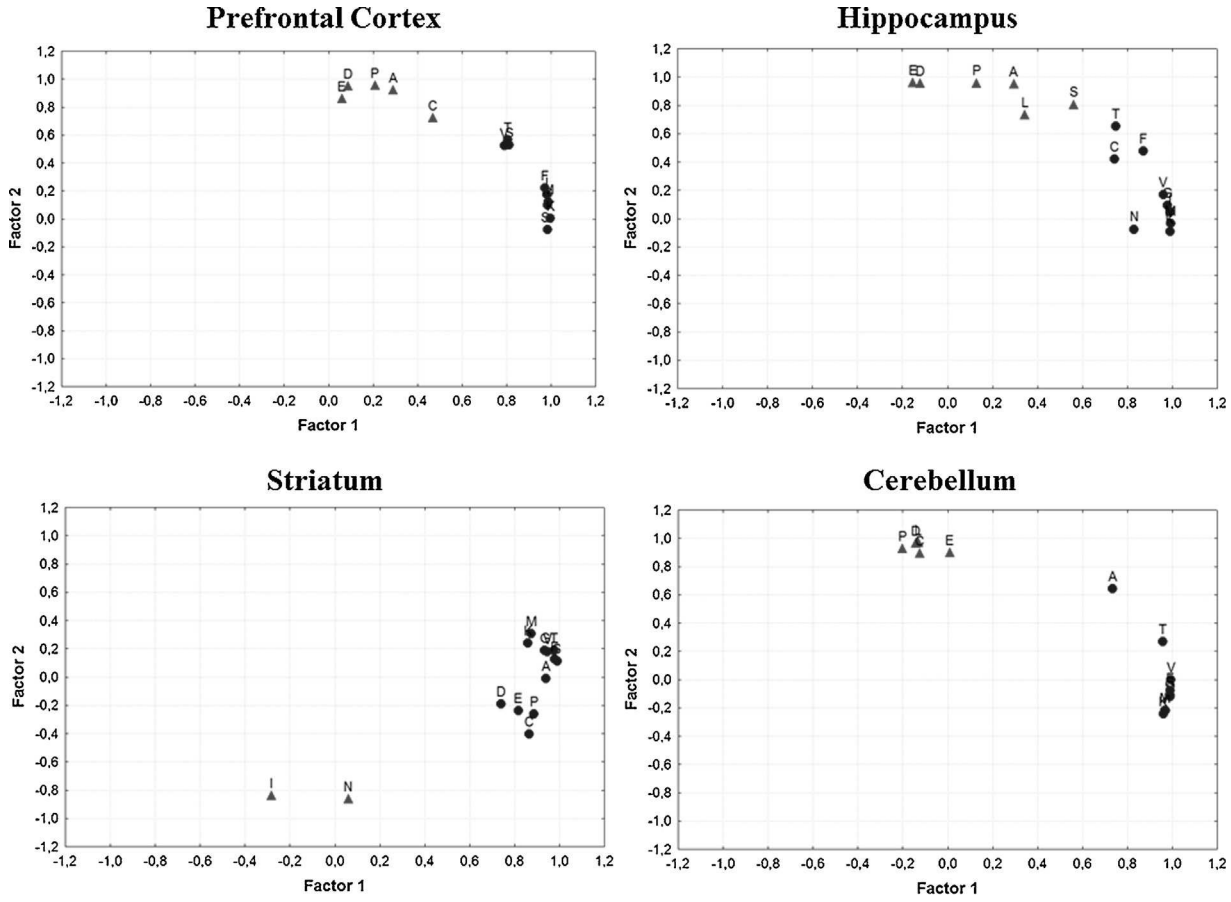

Fig. 9. Factorial analysis of brain levels of amino acids in C57BL/6 mice. Alanine (A), glycine (G), sarcosine (Sr), L-valine (V), L-leucine (L), L-isoleucine (I), L-serine (S), L-threonine (T), L-aspartic acid (D), L-methionine (M), L-proline (P), L-cysteine (C), L-glutamic acid (E), L-phenylalanine (F), L-asparagine (N) and L-lysine (K). The data were extracted by the principal components method; varimax normalization was used as factor loadings and was marked for loadings greater than 0.70.

Our method includes two steps before analysis, extraction and derivatization, which consume 25 and 3 min, respectively. The extract made here used a neutral pH (7.20) with a cocktail of protease inhibitors, which minimize the degradation of the samples, followed by deproteinization with cold methanol. In several methods, the deproteinization process involves homogenization of the tissue in an aqueous strong acid solution and the separation of an insoluble pellet from the supernatant by centrifugation (Shah et al., 2002). This type of preparation can induce hydrolysis of several amino acids, such as asparagine to aspartic acid and glutamine to glutamic acid (Shah et al., 2002). Moreover, BSTFA microwave derivatization requires a shorter time and has lower limits of quantification than other methods (Schwarz et al., 2005; Kaspar et al., 2009; Waldhier et al., 2010; Waterval et al., 2009; Visser et al., 2011). Furthermore, it was demonstrated that a single BSTFA derivatization was useful for the analysis of 16 amino acids in brain samples, which allowed speed and efficiency. However, this method was unable to detect arginine and histidine due to the thermal instability of their derivatives. This method showed good resolution, comparable to those that use N-(tert-butyldimethylsilyl)-N-methyltrifluoro-acetamide (MTBSTFA) for derivatization (Wood et al., 2006), and is a suitable alternative technique for the determination of amino acid levels in brain samples.

In rats and mice, the glutamate and aspartate levels were more concentrated in brain than in serum. Glutamatergic neurotransmission is the most important excitatory pathway in the brain, being involved in several physiological mechanisms such as synaptic plasticity, learning and memory (Shah et al., 2002; Coyle, 2012). Over the last decade, new findings have supported the hypothesis that hypofunction of glutamatergic neurotransmission contributes to the etiology of schizophrenia (Coyle, 2012; Javitt, 2012). Although the role of aspartate in neurotransmission is less studied, several reports have demonstrated its function in neurons. An interesting feature of aspartate is its great affinity for excitatory amino acid transporters, higher than glutamate, and its lower affinity for glutamate receptors, which indicate a neuromodulatory function of this neurotransmitter (Shah et al., 2002).

Proline, which is derived from the amino acid L-glutamate, was also less concentrated in serum than brain structures of rats and mice. In the brain, proline also shows a neuromodulatory function on glutamatergic neurotransmission because proline transporters are expressed on presynaptic neurons of excitatory pathways (Ferreira et al., 2012; Delwing et al., 2007; Cohen and Nadler, 1997a, 1997b). The genetic deficiency on proline catabolism (hyperprolinemia) may cause brain damage and spatial memory deficits (Wyse and Netto, 2011; Bavaresco et al., 2005).

Glycine content in rats and mice was highest in cortex, hippocampus and cerebellum, which is consistent with its role in glutamatergic and glycinergic neurotransmission. Glycine is a co-activator of NMDA receptors and is the key for the involvement of glycine in the pathophysiology of schizophrenia (Shah et al., 2002; Cubelos et al., 2005; Zafra et al., 1995a, 1995b). Furthermore, glycine is the second major inhibitory neurotransmitter in the CNS and has an important role in inhibitory interneurons in the spinal cord (Bowery and Smart, 2006). Glycinergic neurotransmission is also important for sensorimotor control and is widely present in the medial corticohypothalamic tract, in spinal afferents from the

raphe nuclei and reticular formation and in interneurons located in the striatum and substantia nigra (Bowery and Smart, 2006; Rajendra et al., 1997).

We also studied the concentration of sarcosine, an intermediate of the glycine-choline pathway and, although not a neurotransmitter, this molecule plays an important role in the CNS. Sarcosine is a competitive inhibitor of glycine transporter type 1, a co-agonist of NMDA receptor and a weak agonist of glycine receptors (Zhang et al., 2009). It is well known that glycine transporter type 1 inhibitors potentiate the NMDA receptor response in glutamatergic neurotransmission through an increase of the co-agonist glycine in the synaptic cleft (Chen et al., 2003; Lim et al., 2004; Kinney et al., 2003). Based on these pharmacological activities, sarcosine has antischizophrenic and neuroprotective activity (Hsien-Yuan et al., 2008; Tsai et al., 2004; Pinto et al., 2012). The study of this molecule is of great interest to the field of neuroscience because variations in its concentration can affect behavior and brain function in models of schizophrenia.

Another important amino acid for glutamatergic neurotransmission is serine, which was higher in cortex and hippocampus than in serum in both rodents. L-Serine can be converted to D-serine by serine racemase, a brain-enriched enzyme, in neurons in the visual system (Wolosker et al., 2008; Baumgart and Rodríguez-Crespo, 2008). D-Serine acts in glutamatergic neurotransmission by binding to the glycine site of NMDA receptors (Wolosker et al., 2008; Baumgart and Rodríguez-Crespo, 2008). D-Serine modulates NMDA function and is involved in several physiological and pathological processes, such as schizophrenia and neurotoxicity (Wolosker et al., 2008; Baumgart and Rodríguez-Crespo, 2008).

We also studied the concentrations of methionine and cysteine. The first was higher in brain structures than in serum, whereas the second was higher in hippocampus for rats and serum for mice. Methionine is a major methyl donor in the brain and shows some antidepressant properties in rodents, but that mechanism remains unclear (Young and Shalchi, 2005). Cysteine shows two antagonist effects: the first is a neuroprotective effect through glutathione synthesis in neurons, where cysteine serves as an inorganic sulfate for detoxification reactions (Janáky et al., 2000). In contrast, the second effect is neurotoxic, with exogenous administration of L-cysteine evoking cell damage and behavioral deficits in animal models (Janáky et al., 2000). L-Cysteine may play a role as a neuromodulator and has been related to neurological disorders (Janáky et al., 2000).

In rats, we observed that isoleucine was more concentrated in serum than in brain structures, while we observed no differences in mice. In rats, leucine was less concentrated in serum than in brain structures; however, in mice, leucine was most concentrated in the hippocampus. The alterations in the levels of leucine, isoleucine and valine, also known as branched-chain amino acids (BCAA), are closely linked to behavioral disorders and obesity (Coppola et al., 2013). In rats, dietary supplementation of BCAA decreases exploratory behavior in the elevated plus maze test, indicating increased anxiety (Coppola et al., 2013).

In rats, phenylalanine levels were higher in striatum than in hippocampus and serum, whereas in mice, phenylalanine was lower in striatum than in other brain structures. In striatum, accumulation of phenylalanine is associated with the synthesis and degradation of monoamines (Mehta et al., 2005). Acute depletion of tyrosine and phenylalanine disrupts dopamine levels and alters working memory in humans (Mehta et al., 2005). The high concentrations of L-phenylalanine during phenylketonuria (>1.20 mM) cause mental retardation, which is characteristic of the genetic disorder phenylketonuria (Glushakov et al., 2002). These effects occur in part due to the L-phenylalanine inhibition of NMDA receptor current in neurons through the glycine-binding site, which causes depression in glutamatergic synapses (Glushakov et al., 2002, 2005).

Exploratory factorial analysis is a statistical technique that seeks relationship patterns between large numbers of dependent variables. This analysis allows the reduction of the number of dependent variables to a smaller number (factors) and to extract additional information from these hidden factors. In mice, the analysis revealed a dichotomy between the factors in the brain structures, and it was possible to observe an association between the excitatory amino acids (aspartate, glutamate and proline) or structural and inhibitory amino acids (glycine, valine, phenylalanine and lysine). In rats, the excitatory amino acids glutamate and aspartate were associated with all rat brain tissues. The construction of these factors shows how these molecules are related to each other in different parts of the brain, a balance that is crucial to the function of the CNS.

5. Conclusion

In conclusion, this work shows that the determination of amino acid levels in brain samples of Wistar rats and C57BL/6 mice using a gas chromatography–mass spectrometry method provides a cerebral profile of amino acids in brain substrates and serum. The lysis buffer solution showed good sample extraction and enabled parallel analyses by western blotting and GC–MS, and the microwave derivatization allows a faster process. This study shows that this GC–MS method is a reproducible, fast and efficient procedure, permitting multiple analyses with the same samples. This method offers a useful tool for pharmacological and physiological studies to improve the understanding of cerebral chemistry during physiological and pathological conditions.

Conflict of interest

The authors declare that they have no conflict of interest.

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Metabolite Profiling Identifies a Branched Chain Amino Acid Signature in Acute Cardioembolic Stroke

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Background and Purpose—There is limited information about changes in metabolism during acute ischemic stroke. The identification of changes in circulating plasma metabolites during cerebral infarction may provide insight into disease pathogenesis and identify novel biomarkers.

Methods—We performed filament occlusion of the middle cerebral artery of Wistar rats and collected plasma and cerebrospinal fluid 2 hours after the onset of ischemia. Plasma samples from control and patients with acute stroke were also analyzed. All samples were examined using liquid chromatography followed by tandem mass spectrometry. Positively charged metabolites, including amino acids, nucleotides, and neurotransmitters, were quantified using electrospray ionization followed by scheduled multiple reaction monitoring.

Results—The concentrations of several metabolites were altered in the setting of cerebral ischemia. We detected a reduction in the branched chain amino acids (valine, leucine, isoleucine) in rat plasma, rat cerebrospinal fluid, and human plasma compared with respective controls (16%, 23%, and 17%, respectively; $P<0.01$ for each). In patients, lower branched chain amino acids levels also correlated with poor neurological outcome (modified Rankin Scale, 0–2 versus 3–6; $P=0.002$).

Conclusions—Branched chain amino acids are reduced in ischemic stroke, and the degree of reduction correlates with worse neurological outcome. Whether branched chain amino acids are in a causal pathway or are an epiphenomenon of ischemic stroke remains to be determined. **(*Stroke.* 2013;44:1389-1395.)**

Key Words: cerebrospinal fluid ■ liquid chromatography ■ mass spectrometry ■ metabolomics ■ transient ischemic attack stroke

The underlying pathogenesis of acute ischemic stroke remains poorly understood, with a paucity of biological insight translating into useful therapy in patients. Metabolomics is an emerging analytic technology for understanding disease pathogenesis that can be applied to both animal models and patient blood samples. It therefore represents an attractive translational tool to link the biology of model systems to the pathophysiology in patients. Using either nuclear magnetic resonance spectroscopy or mass spectrometry (MS),[1] metabolomics can measure numerous small metabolites simultaneously.[2] MS-based profiling methods include gas chromatography–MS and liquid chromatography coupled to MS, the most common of which is tandem MS (LC-MS/MS).[3] Approaches that use LC-MS/MS are increasingly used because of their sensitivity, flexibility, and quantitative capability for small molecule detection.[2]

Metabolomic profiling has found application in other forms of metabolic stress,[4] including intense exercise,[5] myocardial ischemia,[6] myocardial infarction,[7] and diabetes mellitus,[8–10] but little is known about metabolite changes in the setting of stroke. A common strategy used, in prior metabolomics studies, was to compare the metabolome within subjects, before and after the exposure. However, baseline blood sampling is not feasible in patients with acute stroke. We therefore sought to establish a metabolomic profile in an animal model of ischemic stroke in which baseline sampling is possible, and then integrate the findings with profiling in individuals with acute ischemic stroke. Using a rodent filament occlusion model, we first identified potential candidates whose plasma and cerebrospinal fluid (CFS) were altered. We then evaluated those candidates in an analogous patient cohort in which plasma samples were collected in the acute setting. We hypothesized that we could detect a specific pattern of circulating metabolites that would reflect the chain of metabolic events that occur during cerebral ischemia. Our goal was to apply this new systematic tool as a first step to better understand the biology and pathogenesis of acute ischemic stroke. In doing so, we also explored whether these candidates might serve as potential biomarkers for diagnosis or prognosis.[11–13]

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Methods

Animals

Adult male Wistar rats weighing 275 to 350 g were obtained from Charles River Laboratories (Wilmington, MA). Animals were housed with free access to food and water. The evening before surgery, animals were given nothing per oral to avoid the effect of dietary intake on circulating metabolites. Transient filament occlusion was performed using a 4-0 siliconized suture (Doccol Corp, Sharon, MA) according to standard methods (Methods in the online-only Data Supplement).[14,15] Approximately 250 μL of plasma was withdrawn at baseline and at 2 hours after ischemia onset.

CSF (≈50 μL) was collected from the cisterna magna at 2 hours after ischemia, using a 27-gauge winged needle set attached to a 1 cm^3 syringe.[16] Animals were allowed to recover, and at 24 hours after ischemia, brains were harvested for 2,3,5-triphenyltetrazolium chloride staining to assess the size of stroke.[17] All experiments were approved under an institutionally approved protocol in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Patients

We analyzed EDTA-containing plasma samples collected at a single center, as part of a prospective 2 center biomarker study of acute ischemic stroke (Specialized Programs of Translational Research in Acute Stroke [SPOTRIAS] Network). The SPOTRIAS biomarker study enrolled consecutive patients aged ≥18 years between January 2007 and April 2010, who presented to the Massachusetts General Hospital Emergency Department within 9 hours of symptom onset, with symptoms consistent with ischemic stroke (see Methods in the online-only Data Supplement for additional details of the cohort and the patient data and imaging collection). Ischemic stroke was defined as acute-onset focal neurological deficit with neuroimaging evidence of infarction, or symptom duration >24 hours in the setting of negative diffusion-weighted MRI. Transient ischemic attack was defined as resolution of neurological symptoms within 24 hours that were consistent with a vascular ischemic event (n=18). The designation of "not a stroke" was reserved for subjects with a negative diffusion-weighted MRI who also had an alternative diagnosis for neurological symptoms at discharge (n=14). All subjects or their healthcare proxy provided informed consent, and this study was approved by the local institutional review board.

We applied a case-control design to mirror the animal modeling experiments. We defined 3 groups from the SPOTRIAS biomarker cohort: control, mild, and severe stroke groups. Controls included all subjects with a final diagnosis of transient ischemic attack or absence of stroke (n=32). A similar sized group of mild ischemic stroke was selected from cardioembolic stroke subjects, and 22 sequential subjects with a National Institutes of Health Stroke Scale (NIHSS) ≥4 were used. We also selected sequential subjects with severe cardioembolic stroke, defined as those with an NIHSS ≥15 (n=30). All subjects or their healthcare proxy provided informed consent, and this study was approved by the local institutional review board.

High Performance Liquid Chromatography and Tandem MS

EDTA blood samples were collected and immediately centrifuged to separate cellular material. Aliquots of plasma supernatant were frozen on dry ice and stored at −80°C until analysis. Plasma samples (10 μL) were deproteinized with 90 μL acetonitrile/methanol (3:1; v/v) containing internal standards (valine-d8 [Sigma-Aldrich] and phenylalanine-d8 [Cambridge Isotope Laboratories]). After centrifugation, the extracts were subjected to normal phase hydrophilic interaction chromatography. The chromatography system consisted high throughput screen prep and load autosampler (Leap Technologies, Carrboro, NC) connected to a high performance liquid chromatography pump (1200 Series, Agilent, Santa Clara, CA). MS data were acquired using a 4000 QTRAP triple quadrupole mass spectrometer (Applied Biosystems/Sciex, Framingham, MA) equipped with an electrospray ionization source. Positively charged amino acids, nucleotides, and neurotransmitters were selected for targeted MS/MS analysis using selected multiple reaction monitoring conditions determined previously using reference standards.[6,7]

A total of 68 endogenous metabolites were monitored and detected for each sample. The metabolites were selected on the basis of a broad representation of diverse metabolic pathways as possible, balanced against compatibility with the chromatography and MS ionization method. Deuterated internal standards (valine-d8 and phenylalanine-d8, Cambridge Isotope Laboratories (Andover, MA)) were included in each sample to monitor for quality control. Any sample with internal standard values ≥2 SD were excluded from peak integration and further analysis. In addition, pooled plasma samples were interspersed within each analytic run at standardized intervals, enabling the monitoring and correction for temporal drift in MS performance. Each of these samples were prepared, extracted, and processed as separate 10 μL aliquots from a larger pool of normal human plasma. Replicate injections of pooled plasma demonstrated that 50% of the analytes had a coefficient of variation ≤5% (including the branched chain amino acids [BCAA]), 69% of the analytes had a coefficient of variation ≤10%, and 91% had coefficient of variation ≤20%, which is consistent with prior studies.[9]

Statistical Analysis

Univariate Analysis

Differences in clinical and laboratory continuous variables were compared using Student *t* test or Mann–Whitney test, as appropriate. Categorical variables were compared using Fisher exact test. For the metabolite analysis in the animal samples, we used an uncorrected *P* value threshold of 0.05, using Mann–Whitney or Student *t* test, depending on data normality. In this exploratory phase, no correction for multiple comparisons was made.

In the human cohort analysis, we used a similar approach to our prior studies[5] and applied the Benjamini–Hochberg procedure[18] to limit the false discovery rate to q<0.1, which corresponded to a threshold of $P<0.015$. This would be expected to yield ≈1 false-positive discovery in 68 metabolites analyzed, assuming independent hypotheses. Moreover, this threshold also approximates the Bonferroni correction of the combined probability between the discovery cohort ($P<0.05$) and the human validation cohort ($P<0.015$) (ie, $0.05\times0.015=7.5\times10^{-4}$, whereas Bonferroni correction=$0.05/68=7.4\times10^{-4}$). Although many metabolites were associated with predefined groups (eg, amino acids, tryptophan derivatives, nucleotide metabolites, etc.), this is a conservative estimate because the number of independent tests was substantially lower than the nominal ones. Statistical analyses were performed using the STATA statistical software (release 12) or JMP 10 Pro (SAS Institute, Cary, NC).

Multivariate Analysis

To uncover the multivariate structure within the human data set, we performed principal component analysis and partial least-squares discriminant analysis using MetaboAnalyst 2.0 (Edmonton, BC, Canada).[19] Because each method provides slightly different insight into high-dimensional data, we performed to highlight the metabolites in common (see Methods in the online-only Data Supplement for further details).

Results

Using LC-MS/MS, we first examined serial blood samples after filament occlusion in a rat model of ischemic stroke. We collected plasma at baseline and 2 hours after ischemia, as well as CSF at the 2-hour time point. In pilot experiments, the placement of a laser Doppler flowmetry probe led to poor recovery of CSF (data not shown). Exploiting the variability in stroke volume that would occur in the absence of Doppler flowmetry, we designed our experiment as a comparison among sham, small stroke, and large stroke animal cohorts. Of 23 animals, 2 died acutely and the volume of infarct could

not be determined. Another animal assigned to the middle cerebral artery occlusion group had no infarction at 24 hours and was therefore excluded. The remaining 20 animals were included in the analysis: 7 sham-operated animals, 6 animals with small infarction (stroke volume 9±5%), and 7 animals with large infarction (stroke volume 29±5%; Table I in the online-only Data Supplement).

We measured a total of 68 metabolites in baseline and 2-hour follow-up plasma samples, and results were analyzed as a percent change from baseline, which adjusts for within animal variation. To eliminate any nonspecific effects of the operative technique, we compared percent metabolite changes in stroke animals to those in sham-operated animals. From baseline to 2 hours after stroke, there was a significant decrease in the concentration of BCAA leucine, isoleucine, and valine in the large stroke group (P=0.003, 0.01, and 0.04, respectively). BCAA are coordinately regulated, and the levels change in conjunction with each other.[20] Accordingly, a composite measurement of the BCAA showed a 16±6% decrease in large stroke (P=1×10^{-5}; Figure 1D) and a nonsignificant trend in small stroke. Several other metabolites were altered in a dose-dependent manner in small and large stroke. These included stepwise increases in xanthosine (+57%; P<0.001), carnosine (+71%; P<0.005), and glutamate (+40%; P=0.01), and decreases in niacinamide (−31%; P=0.02) and phenylalanine (−18%; P<0.01) relative to sham-operated animals.

We also measured the same metabolites in the CSF obtained through cisterna magna puncture at 2 hours after onset of ischemia. Because the concentration of most metabolites in CSF is lower than in plasma, we excluded any CSF samples with visible blood contamination (Methods and Figure I in the online-only Data Supplement). Figure 2A through 2D shows that the individual BCAAs had a consistent trend toward a decrease (leucine −21%, P=0.06; isoleucine −23%, P=0.14; valine −22%, P=0.11). Moreover, a composite of BCAA demonstrated a decrease of 23±9% compared with sham CSF (n=5 for each group; P<0.005). Other significantly altered CSF metabolites included an accumulation of xanthosine (102%, P=0.01) and lysine (18%, P=0.02).

On the basis of the animal studies, concordant metabolite changes between plasma and CSF included valine, leucine, isoleucine, and xanthosine. We next evaluated whether these candidate metabolites were altered in the plasma of patients with acute stroke to determine whether these metabolite changes represented a common alteration. We obtained plasma samples from a cohort of patients in whom blood was collected acutely, shortly after presentation to the emergency department. We selected a subset of subjects to coincide with the animal modeling design, which included a control group (patients with a diagnosis of transient ischemic attack or absence of stroke), a group with mild stroke (patients with an NIHSS 4–5), and a severe stroke group (NIHSS 15–19). To limit potential heterogeneity, we focused on subjects with a cardioembolic cause of stroke. The clinical characteristics of the cohort are listed in Table 1. As would be expected, the stroke group had an older age and higher rates of atrial fibrillation compared with the control group. In addition, the large stroke group had a higher acute stroke volume, higher acute NIHSS, and worse 3-month neurological outcome as compared with the small or control groups.

We analyzed plasma samples obtained within 6±2 hours from the last seen well time, using our metabolomics method. Heat map correlation analysis confirmed a close association of the BCAAs (Figure 3, top right), consistent with the animal modeling data and with the known coordinated metabolism of these amino acids.[20] Analysis of individual metabolites showed that leucine, isoleucine, and valine were all decreased in stroke compared with control, and to a greater extent in large compared with small stroke (P<0.01 for each; Figure 4). Similarly the composite BCAA score demonstrated a 9±17%

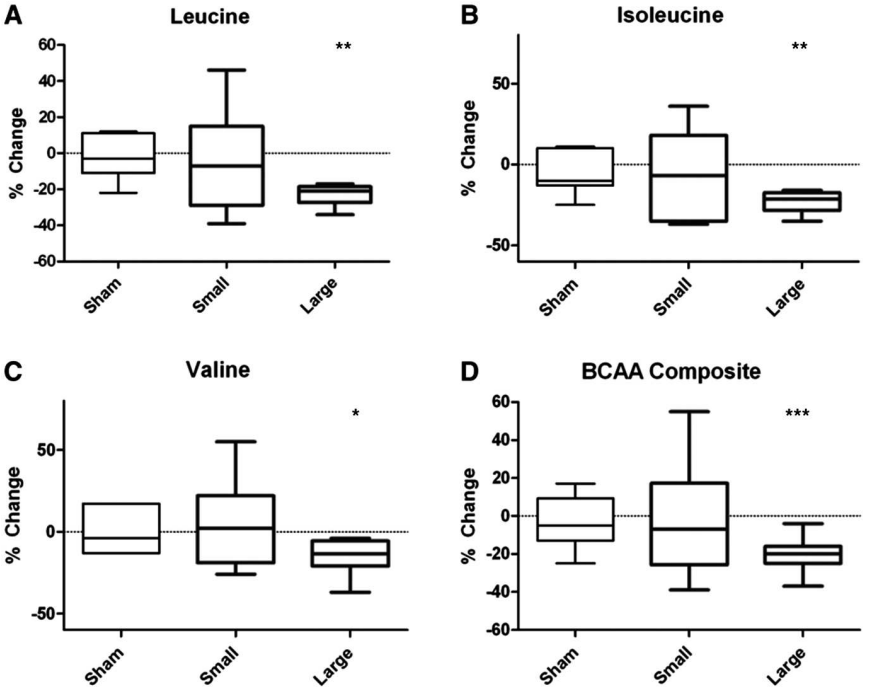

Figure 1. A–D, Rats subjected to filament occlusion of the middle cerebral artery had plasma collected at baseline (just before filament occlusion) and 2 hours after stroke. The concentration of branched chain amino acids (BCAA) were diminished from baseline to 2 hours after stroke. *P<0.05, **P<0.01, and *** P<0.001.

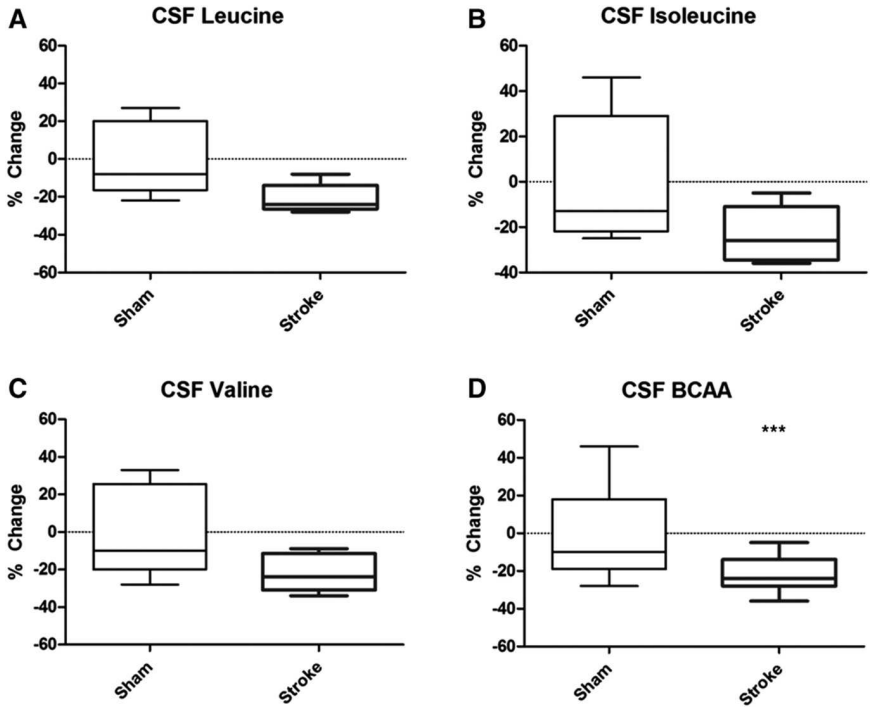

Figure 2. A–D, Change in branched chain amino acids (BCAA) in cerebrospinal fluid (CSF) from baseline to 2 hours after stroke (n=5 for each group). Each individual BCAA showed a trend toward a decrease, P=0.06, 0.14, and 0.11, respectively, whereas the composite BCAA, xanthosine and lysine were significant, $^{*}P$<0.01, $^{**}P$<0.01.

decrease in small stroke (P=0.03) and a 17±23% decrease in large stroke (P=1.1×10^{-5}). Table 2 provides a complete list of all metabolites that were altered in the setting of ischemia when compared with control patients. In addition to novel metabolites, we found that glucose showed a significant increase in stroke compared with control, which is concordant with the well-described phenomenon of acute stress hyperglycemia.[21–23]

To further simplify the high-dimensional metabolomics data, we next performed principal component analysis. This approach consolidates data into fewer metabolite clusters, which maximally explain the variance in the data.[19] Intriguingly, the first principal component (principal component, which explained 20% variance in the data; Figure II in the online-only Data Supplement for score and loading plots) contained the BCAA metabolites. In addition to leucine, valine, and isoleucine, the first principal component also included tyrosine, lysine, and methionine. Comparing the individual subjects' scores, the first principal component also distinguished cases from controls (P=0.020 comparing control versus all stroke and P=0.011 for control versus large stroke).

Next, we performed partial least-squares discriminant analysis, which is a method of supervised classification that is designed to highlight metabolite differences between cases and controls. This technique is commonly used in metabolomics studies for biomarker discovery because it emphasizes the distinction between the 2 classes.[19] The metabolites that contributed the greatest discrimination between stroke and controls were similar to our univariate analysis presented in Table 2. These included the BCAAs, carnitine, threonine, histidine, and glucose (Figure III in the online-only Data Supplement). Validation of the model was confirmed using cross-validation and permutation testing (P<0.01; Figure III in the online-only Data Supplement).[19,24]

Having confirmed that BCAA were altered acutely in stroke, we next explored its association with imaging and clinical measures. Because the magnitude of BCAA change seemed to correlate with size of stroke in the animal model, we evaluated the correlation between BCAA and diffusion-weighted volume in the patient cohort. There was a nonsignificant trend in association between admission infarct volume and BCAA (r=–0.18; P=0.11). On the contrary, a lower concentration of BCAA was associated with increased age (r=–0.26; P=0.02), female sex (P<0.001), and worse outcome at 3 months (modified Rankin Scale, 3–6; P=0.002). Because age and sex are also recognized predictors of worse neurological outcome,[25,26] we explored whether BCAA predicted outcome independently of age and sex. Although the

Table 1. Clinical Characteristics of the Stroke Cohort

	TIA (N=32)	Stroke (N=52)	P Value
Female, N, %	16 (50%)	22 (42%)	0.51
Age±SD, y	66±16	75±10	<0.01
Admission temp, °F±SD	98.3±0.7	98.2±1.1	0.72
CAD, N, %	9 (28%)	17 (33%)	0.81
HTN, N, %	25 (75%)	45 (87%)	0.37
DM2, N, %	11 (34%)	12 (23%)	0.32
HL, N, %	15 (47%)	23 (44%)	0.83
Afib, N, %	5 (16%)	33 (63%)	<0.001
		Small	Large	
NIHSS, median [IQR]	3 [1, 8]	4 [4, 5]	17 [15, 19]	<0.001
DWI volume, median [IQR]	0 [0, 3]	3 [1, 15]	25 [11, 59]	<0.001
3 Months mRS, 0–2, %	23 (79%)	11 (65%)	5 (23%)	<0.001

Afib indicates atrial fibrillation; CAD, coronary artery disease; DM2, diabetes mellitus 2; DWI, diffusion-weighted imaging; HL, hyperlipidemia; HTN, hypertension; IQR, interquartile range; mRS, modified Rankin Scale; NIHSS, National Institutes of Health Stroke Scale; TIA, transient ischemic attack.

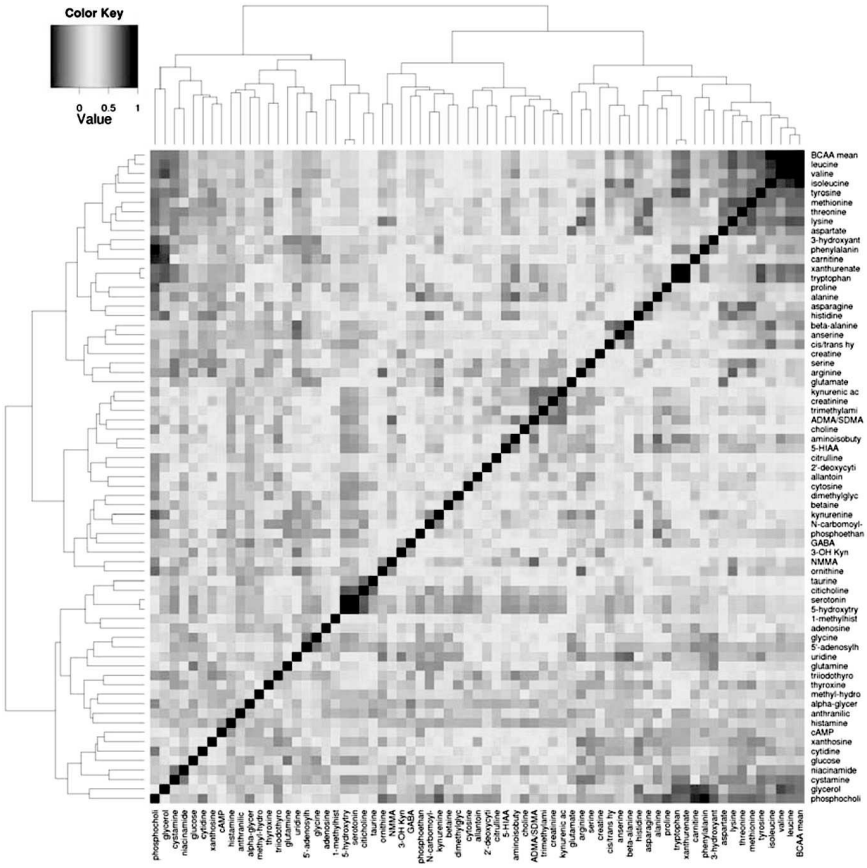

Figure 3. Heat map representation of metabolites highlights the tight correlation of the branched chain amino acid (BCAA), which are located in the top right. The heat map is generated from 52 patients with acute stroke who had blood samples drawn at 6±2 hours from the last seen well time. Analytes that are positively correlated are represented in red, whereas compounds inversely correlated are represented in blue. ADMA indicates asymmetric dimethylarginine; GABA, gamma aminobutyric acid; HIAA,5-hydroxyindoleacetic acid; and SDMA, symmetric dimethylarginine.

cohort was limited in size and stratified on the basis of stroke severity, we performed exploratory multivariable logistic regression and found that BCAA remained an independent predictor of outcome (P=0.04) after adjusting for age and sex.

Discussion

Using metabolomics, we have identified specific circulating metabolites that are altered in the setting of cerebral infarction. On the basis of our systematic analysis in a well-controlled

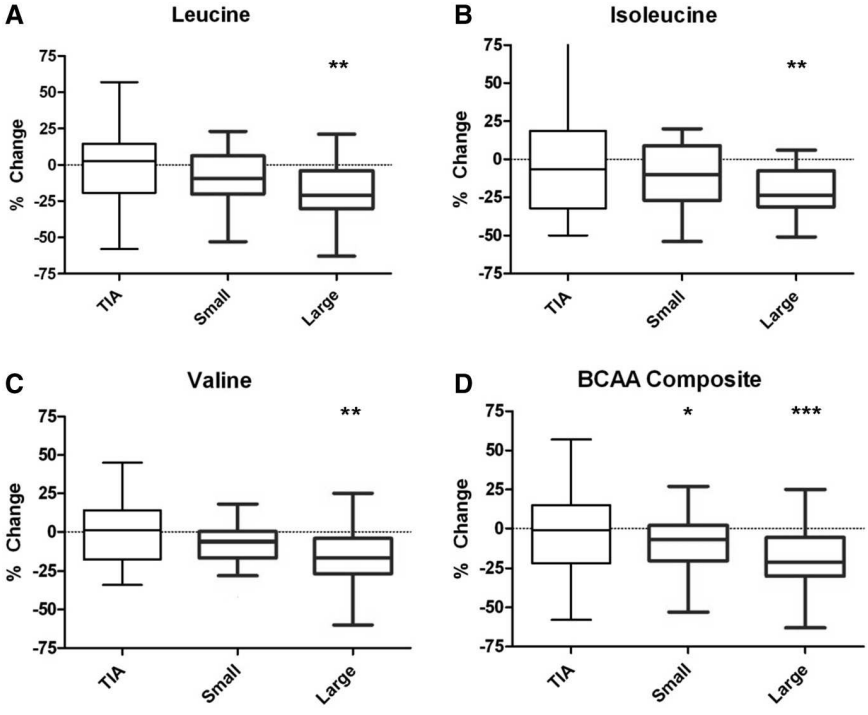

Figure 4. A–D, The concentration of plasma branched chain amino acids (BCAA) in patients with stroke is reduced when compared with control subjects at the time of acute presentation. *P<0.05, **P<0.01, and ***P<0.001.

Table 2. All Metabolites Significantly Changed in Human Stroke Subjects Compared With Control

Metabolite	Fold Change In Stroke	*P* Value	BH Procedure, Q<0.1
Carnitine	0.89	0.001	0.0015
Threonine	0.80	0.002	0.0029
Histidine	0.83	0.003	0.0044
Glucose	1.42	0.0057	0.0059
Valine	0.88	0.007	0.0074
BCAA mean	0.86	0.008	0.0088
Methionine	0.82	0.009	0.0103
Leucine	0.86	0.009	0.0117
Glycine	0.82	0.0131	0.0132
Proline	0.89	0.017	0.0147
Lysine	0.86	0.025	0.0162
Cysteamine	0.56	0.027	0.0177
Isoleucine	0.85	0.028	0.0191
Uridine	0.81	0.033	0.0258
5′-Adenosylhomocysteine	0.84	0.036	0.0221
Creatinine	0.89	0.039	0.0235
N-carbamoyl-β-alanine	1.33	0.041	0.0250
cis/trans Hydroxyproline	0.73	0.041	0.0265
Asparagine	0.89	0.043	0.0279

The false discovery threshold based on the Benjamini–Hochberg (BH) procedure[18] is indicated by the bold line. For completeness, additional metabolites that exceed this threshold, but with an uncorrected *P*<0.05, are listed below the bold line.

BCAA indicates branched chain amino acids.

animal model and linking those findings to patient samples in the acute setting, we have identified a small and interrelated subset of metabolites. Our data demonstrate a reduction in the concentration of BCAAs that associates with stroke severity and worse neurological outcome. Although our data do not point to an underlying biological mechanism, they focus future experiments on investigating candidate pathways that relate to BCAA. The notion that BCAA play an important role in the metabolic response to disease is supported by evidence of its alteration in other illnesses. For example, BCAA is reduced in critical illnesses, such as sepsis, trauma, and burn injury.[27–29] BCAA is also associated with the risk of incident diabetes mellitus[9] and can induce insulin resistance,[8] further suggesting a role in metabolic homeostasis. Perhaps, most interestingly, BCAA are altered also in heart disease,[30] suggesting that these amino acids play a critical role in bioenergetic homeostasis. Whether BCAA represent a novel link between cardiovascular and cardioembolic cerebrovascular diseases requires further investigation.

In addition to their potential role in systemic disease states, BCAA also serve a unique role in the brain.[31,32] For example, BCAA are integral to the glutamate/glutamine cycle between astrocytes and neurons, which is critical for the efficient uptake of glutamate during excitatory neuronal signaling.[31] Intriguingly, inhibition of the first step of BCAA catabolism with gabapentin reduces brain glutamate concentration.[31] Gabapentin has been reported to reduce stroke volume in a rodent model,[33] and 1 possibility is that it may do so by limiting glutamate concentration and subsequent excitotoxicity. Although our rodent data showed an accumulation of glutamate, we did not detect a similar change in the patients. Whether this reflects inadequate power or greater complexity in the human cohort requires further study. Alternatively, the reduction in BCAA level may reflect a metabolic pathway leading to consumption or sequestration in a tissue compartment other than blood or CSF. BCAA are also known to have roles in protein metabolism and in catabolic energy metabolism.[20] These putative mechanisms are not mutually exclusive, and, indeed, systemic BCAA levels have been shown to influence brain neurotransmitter levels.[32] Nevertheless, our data raise the possibility that manipulation of BCAA may influence outcome. Future studies that focus on whether BCAA are causally related to cerebral ischemia, such as through supplementation and pharmacological inhibition, will help determine whether BCAA holds promise as a therapeutic target.

Our analysis in rodents and patients identified additional candidate metabolites, which were not shared in common between the 2 (Table 2 and Results). The similarities and differences between rodent model systems and patients are an area of substantial importance for translational therapy. Metabolomics is a technique that allows direct comparisons between the model systems and patients in a way that was not previously available. Although our findings with BCAA highlight that there are similar biological pathways in rodents and patients, the differences may offer some caution. Nevertheless, our data point to one approach to explore these similarities and differences systemically, both of which are important for novel target discovery. There is little prior metabolomics analysis of stroke, with the exception of an nuclear magnetic resonance–based study in a cohort of lacunar stroke subjects,[34] which analyzed blood samples collected within 72 hours of stroke onset. Of the overlapping metabolites in common with our method, valine was diminished in lacunar stroke, although leucine and isoleucine were not.[34] The apparent differences may reflect the increased sensitivity of LC-MS/MS compared with nuclear magnetic resonance, differing metabolomes based on stroke subtype, differences in control selection, and potentially in the timing of the blood draw.

Our study has several strengths. We used a carefully controlled model system to establish a metabolite profile and then compared it to a well-phenotyped patient cohort. We used a metabolomics technique that is well validated and possesses excellent quantitative capability and reproducibility. The patient samples were obtained in the hyperacute phase and compared with a control group of stroke mimics. However, there are several limitations to our analysis. We used a targeted metabolomics approach, which identifies a limited set of metabolites rather than a comprehensive list of known and unknown peaks. It is therefore possible that additional metabolite changes occur that we cannot detect with our current method. LC-MS/MS–based metabolomics also has limited throughput capability. Nevertheless, we have selected key sentinel metabolites that are central to several important biochemical pathways, including amino acids, nucleotides, and selected neurotransmitters. Although our data point toward a key role for BCAA in stroke, our correlation and

multivariate regression must be interpreted with caution in a small patient cohort. Most importantly, validation in a larger cohort that includes all stroke subtypes with a broad range of stroke severity will be necessary to confirm our findings and determine whether BCAA holds promise as a clinically useful biomarker or a therapeutic target.

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