Talanta 170 (2017) 63–68

Contents lists available at ScienceDirect

Talanta

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

Capillary electrophoresis tandem mass spectrometry determination of glutamic acid and homocysteine's metabolites: Potential biomarkers of amyotrophic lateral sclerosis

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ARTICLE INFO

Keywords:
Capillary electrophoresis-tandem mass spectrometry
Target metabolomics
Amino acids
Amyotrophic lateral sclerosis
Protein precipitation
Sample preparation

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that affects both lower and upper motor neurons, leading to muscle atrophy, paralysis, and death caused by respiratory failure or infectious complications. Altered levels of homocysteine, cysteine, methionine, and glutamic acid have been observed in plasma of ALS patients. In this context, a method for determination of these potential biomarkers in plasma by capillary electrophoresis tandem mass spectrometry (CE-MS/MS) is proposed herein. Sample preparation was carefully investigated, since sulfur-containing amino acids may interact with plasma proteins. Owing to the non-thiol sulfur atom in methionine, it was necessary to split sample preparation into two methods: *i)* determination of homocysteine and cysteine as S-acetyl amino acids; *ii)* determination of glutamic acid and methionine. All amino acids were separated within 25 min by CE-MS/MS using 5 mol L^{-1} acetic acid as background electrolyte and 5 mmol L^{-1} acetic acid in 50% methanol/H_2O (v/v) as sheath liquid. The proposed CE-MS/MS method was validated, presenting RSD values below 6% and 11% for intra- and inter-day precision, respectively, for the middle concentration level within the linear range. The limits of detection ranged from 35 (homocysteine) to 268 nmol L^{-1} (glutamic acid). The validated method was applied to the analysis of plasma samples from a group of healthy individuals and patients with ALS, showing the potential of glutamic acid and homocysteine metabolites as biomarkers of ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease caused by progressive loss of motor neurons in the cerebral cortex, brainstem and spinal cord [1]. About 5–10% of all ALS patients are familiar, and the remaining cases have unknown cause [2]. Currently, the diagnosis is still based on clinical findings (the El Escorial Criteria), which delays the diagnosis to nearly one year [3,4]. Considering that most patients die within 2–4 years of disease onset, such delay is unacceptably long, and new diagnostic approaches should be conceived.

Intensive efforts have been dedicated towards finding biomarkers of ALS aiming to diagnose the disease in early stages. Recently some studies have shown altered levels of homocysteine (Hcy) in biological fluids of patients with ALS [5–7]. Hcy is a non-proteinogenic sulfur-containing amino acid, which plays an important role in living organisms. Its metabolism is intermediate to re-methylation to methionine (Met) and transsulfuration to cysteine (Cys) via cystathionine pathway [8].

Altered levels of glutamic acid (Glu) have been related to ALS as well [9,10]. This amino acid is an important excitatory neurotransmitter, but its excessive stimulation at post synaptic receptors can lead to neurotoxicity due to elevated flow of calcium into neurons. Therefore, the high concentration of calcium inside neurons activate enzymes that initiate the apoptosis cascade [11].

Determination of amino acids in body fluids is usually based on

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http://dx.doi.org/10.1016/j.talanta.2017.03.103
Received 9 February 2017; Received in revised form 29 March 2017; Accepted 30 March 2017
Available online 01 April 2017

separation methods such as high- or ultra-performance liquid chromatography (HPLC or UPLC) and capillary electrophoresis (CE) with fluorescence or UV detection [12–16]. As long as Glu, Met, Cys, and Hcy scarcely absorb UV radiation and, in general, do not present fluorescence, a derivatization step is required prior to the analysis. Usually, a specific probe containing a chromophore or fluorophore reacts with the amino- or carboxylic group, and, in the case of amino thiols such as Hcy and Cys, it reacts with the thiol group as well [14,17,18]. Furthermore, when amino- or carboxylic group is derivatized to determine amino thiols, the thiol group must be protected against oxidation or formation of side products [12].

To circumvent the derivatization step, detectors based on different analytes properties have been used for determination of amino acids. Examples include the capacitively coupled contactless conductivity detector (C^4D), refractive index detector (RID), and mass spectrometry detection (MS) [19–23]. Amino acids have already been determined in body fluids by CE-C^4D [24]. C^4D detects analytes based on the difference of mobility of the species passing through the detector. For this reason, it is a universal detector, but other ions commonly present in body fluids – such as inorganic ions, may interfere with proper detection. The coupling of CE and MS combines both the high efficiency of separation and low sample consumption from CE and the high sensitivity and structural information from MS, resulting in one of the best available tools for the determination of charged metabolites in body fluids. Several other methods for determination of amino acids in body fluids have been reported [25]. However, the developed methods always treat amino acids and amino thiols separately, because of the rapid and spontaneous oxidation of the thiol group [12].

Therefore, the main goals of this study are providing a trustworthy methodology for the determination of Glu, Hcy, and its metabolites in plasma. Afterwards, they were assessed as potential ALS biomarkers. Hcy has been previously determined in plasma by CE-MS after protein precipitation with methanol and reduction of disulfide bond with mercaptoethanol [26]. However, in the current study, an alternative approach to determine amino thiols and amino acids in plasma is presented, including protein precipitation with dithiothreitol (DTT) and acetonitrile. This strategy is often used for depletion studies in proteomics [27–31], and now it could be successfully applied in target metabolomics. The developed method was applied to the analyses of real samples from both healthy subjects and ALS patients.

2. Materials and methods

2.1. Materials and reagents

Homocysteine (purity≥98%), cysteine hydrochloride (purity≥98%), glutamic acid (purity≥99%), methionine (purity≥98%), glycine-2-^{13}C (purity 99% 13 C), dithiothreitol (purity≥99%), iodoacetic acid (purity≥98%), acetic acid (≥99.7%), methanol and acetonitrile were purchased from Sigma-Aldrich (USA). Individual stock solutions of amino acids at a concentration of 10 mmol L^{-1} were prepared in deionized water (18 MΩ cm) obtained from a Millipore Milli-Q water purification system (Molsheim, France), and stored at −20 °C. Working solutions of individual amino acids and their mixtures were prepared daily by appropriate dilution of the stock solutions with deionized water. Working solutions of DTT and iodoacetic acid (IAA) at a concentration of 500 mmol L^{-1} were prepared daily in deionized water or NaOH (1 mol L^{-1} solution for HPCE, Agilent Technologies, Waldbronn, Germany), respectively. The background electrolyte (BGE) was composed of 5 mol L^{-1} acetic acid and was prepared daily. The sheath liquid was composed of 5 mmol L^{-1} acetic acid in 50% methanol (v/v) and was prepared on a weekly basis.

Table 1
MS/MS acquisition parameters for the identification and quantification of amino acids.

Amino acid	Precursor Ion (*m/z*)	Fragment Ions (*m/z*)	Collision Voltage (V)	Fragmentor Voltage (V)
Glycine ^{13}C[a]	77	49[b] 59	3	70
Methionine	150	104[b] 133	7	70
Glutamic acid	148	84[b] 130	8	90
S-acetyl-homocysteine	194	148[b] 134	10	100
S-acetyl-cysteine	180	163[b] 107	10	100
Homocysteine	136	90	8	90
Cysteine	122	76	8	90

[a] internal standard (IS).
[b] transition used for quantification.

2.2. Instrumentation and analytical conditions

All CE-MS experiments were performed in an Agilent CE 7100 equipment interfaced to a 6430 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) with an electrospray source (ESI) assisted by sheath liquid interface. The sheath liquid was delivered by a 1260 Infinity isocratic pump (Agilent Technologies, Santa Clara, CA, USA) at a flow rate of 6 μL min^{-1}. Nitrogen was used as nebulizer and carrier gas at a pressure of 34.5 kPa and a flow rate of 6 L min^{-1} at 160 °C, respectively. The ESI source operated in positive mode by applying +4.5 kV. The equipment was operated at selected reaction monitoring (SRM) mode using two mass transitions for each amino acid. The mass transitions, fragments, and cell collision voltages are listed in Table 1. The most intense fragment was used as quantification ion, while the second one was used as confirmation ion. Dwell time was 50 ms.

The CE separations were carried out in uncoated fused-silica capillary with total length of 60 cm, inner diameter of 50 μm and outer diameter of 360 μm (Agilent Technologies, Redmond, OR, USA). New capillaries were conditioned with 1 mol L^{-1} NaOH for 30 min, water for 10 min and BGE for 30 min. Before each run the capillary was conditioned with 1 mol L^{-1} NaOH for 100 s, water for 60 s, and BGE for 100 s. The ESI source was turned off during conditioning step. The samples were introduced hydrodynamically by applying a pressure of 50 mbar for 10 s. The CE capillary was kept at 20 °C during CE runs and a voltage of +25 kV was applied.

2.3. Sample preparation

Blood samples from 20 healthy volunteers and 39 patients with ALS (Table 2) were collected in vacuum tubes containing EDTA solution at the ALS Outpatient Clinic at UNICAMP Hospital (UNICAMP,

Table 2
Information about subjects used in the study.

	Healthy controls	Patients with ALS
Total	20	39
Men	10	22
Women	10	17
Age	23 ± 3 years*	54 ± 12 years*

* There is a significant age difference between healthy controls and patients with ALS.