RESEARCH ARTICLE

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High-throughput and high-sensitivity capillary electrophoresis–mass spectrometry method for sulfur-containing amino acids

Jieyu Zhao^{1*}, Bing Dong¹, Yanni Zhao², Jun Yang¹, Pei Pei¹, Xianbing Ji¹ and Yulian Quan¹

Abstract

Biological thiol amino acids have been suggested as biomarkers for pathological changes because they are reactive chemicals that participate in various physiological processes. In this study, multisegmented injection capillary electrophoresis–mass spectrometry with online sample preconcentration was used for analysis of thiol amino acids and intermediates of sulfur metabolism in human glioma cell line U-251 with high accuracy, throughput, and sensitivity. This was achieved using multiple, large-volume injections for online sample preconcentration. The 16 intermediates of sulfur metabolism had a good linear correlation coefficient range of 0.984–1 and the limit of detection range was 1.4–203.9 ng/mL. The recovery ranges of most amino acids were 88.1–114.5%, 89.0–104.3%, and 76.9–104.5% at 0.3, 0.75, and 1.5 µg/mL, respectively. The relative standard deviation ranges for the inter- and intra-day precision were 1.8–10.7% and 4.3–18.8%, respectively. Compared with the traditional injection method, the analytical time for compounds in sulfur metabolism was reduced to 4 min/sample, the method throughput was enhanced five times, and the sensitivity was increased 14.4–33.1 times. Customized injection sequences were applied in experimental optimization. The developed method simplified the experimental optimization to one injection and is suitable for the analysis of sulfur metabolites in biological samples and has high sensitivity, throughput, speed, and accuracy.

Keywords: Capillary electrophoresis–mass spectrometry, Amino acid, Online preconcentration, Large volume injection, Multisegmented injection

Introduction

Sulfur-containing amino acids are important for maintaining cell balance and functions through regulation of cellular redox state and reactive oxygen species to eliminate free radicals and detoxify toxic compounds (Ward and DeNicola 2019; Rouhier et al. 2015; Oliveira and Laurindo 2018; Turell et al. 2013; Go and Jones 2013). S-Adenosylhomocysteine (SAH) is an active methyl group donor and important intermediate of sulfur homeostasis. The transulfuration pathway contains the convertion of methionine to homocysteine, which

*Correspondence: zhaojieyu2004@126.com

¹ Department of Environmental Engineering, Hebei University

of Environmental Engineering, Qinhuangdao, China

Full list of author information is available at the end of the article



is required for protein and DNA synthesis. Thiol balance is the important intersection of the folic acid cycle and one carbon metabolism which are closely related to human pathologies. A sulfur imbalance can produce a variety of metabolic disorders and human diseases (Townsend et al. 2004; Azad et al. 2020). For example, glutathione (GSH) and its disulfide are prevalent antioxidants in the defense against oxidative stress and an indicator of cell pathogenesis (Wojsiat et al. 2018). Both cysteine (Cys) deficiency and accumulation are related to human diseases (Poole 2015; Biteau et al. 2003). Homocysteine is a specific risk marker for cardiovascular disease. Increased in plasma Homocysteine and low serum folic acid and vitamin B₁₂ concentrations might be correlated with Alzheimer's disease in some populations (Cai et al. 2016; Kim et al. 2018). In addition, some

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cancer cells express an unusual dependence on methionine metabolism for growth (Hoffman et al. 2019).

Sulfur's amino acids are usually high polar, present in low concentration, and unstable in biological samples. For example, cysteine is easily transformed to cystine in neutral and basic conditions, Consequently, accurate measurement of cellular thiol-containing amino acid concentrations is challenging because of interference (Comini 2016). Thus, a simple and rapid approach that has high throughout, high sensitivity and is interference-free is required for biological, clinical and pharmacological analyses There are already many analysis methods for sulfur's amino acids, such as capillary electrophoresis (CE) (Hodáková et al. 2015), liquid chromatography-mass spectrometry (LC-MS) (Ortmayr et al. 2015; Sun et al. 2016; Isokawa et al. 2016; Tang et al. 2015; Onozato et al. 2020) and nuclear magnetic resonance spectroscopy (NMR) (Yang et al. 2018). However, because sulfur-containing amino acids are unstable and interference from derivatives and matrix components is common, false positive results are often obtained and the accuracy is affected. Consequently, samples require pre-column derivatization and ion-exchange chromatography for detection. These steps lengthen the analysis time and limit the throughput. Sun et al. developed a chemical derivatization method for LC-tandem MS (Sun et al. 2016), but the separation time was 10 min and only five thiol compounds could be determined simultaneously.

Injection of multiple sample segments from hydrodynamic injection within a single capillary offers a convenient approach to improve sample throughput in CE (Kuehnbaum et al. 2013). CE can reduce ion suppression resulted from highly saline biological samples and is a versatile experimental approach for method validation, accurate qualification and signal pattern recognition (Kuehnbaum et al. 2013; Azab et al. 2020). Furthermore, CE-MS is well-suited for volume-limited samples, especially for single-cell analysis (Nevedomskaya et al. 2010). However, due to the small sample injection volume and post-capillary dilution, the sensitivity of CE-MS is far lower than LC-MS (Kuehnbaum et al. 2013). The pH difference between the sample and background electrolyte (BGE) zone can be tailored so that analytes stack online without any derivatization or interference. Numerous applications have been reported recently (Kitagawa and Otsuka 2014), including amino acids (Britz-McKibbin and Terabe 2003), peptides (Su and Yu 2009), hormones (Bai et al. 2014), nucleotides (Feng and Zhu 2006), proteins (Janini et al. 2003) and other metabolites (Ptolemy and Britz-McKibbin 2008).

Here, we report a sensitive, simple, and high-throughput method for thiol amino acids and intermediates of sulfur metabolism without derivatization using large volume injections and online sample preconcentration.

Materials and methods

Chemicals and materials

GSH, γ -glutamyl-cysteine, Cys, homocysteine, cystathionine, cystine, methionine, SAH, S-carboxymethylcysteine, S-lactoylglutathione, aspartic acid, asparagine, glutamic acid, serine, valine, 1-aminocyclopropanecarboxylic acid, formic acid, and ammonium acetate were obtained from Sigma-Aldrich (St. Louis, MO). All reagents and solvents were of either analytical or HPLC grade.

Sample preparation

Human glioma cell line U251 was purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), and maintained in culture bottles in RPMI medium supplemented with 10% (v/v) fetal bovine serum at 37 °C in a humidified incubator containing 95% air/5% CO₂. After reaching a density of 10^5 cells/mL, the cells were frozen in liquid nitrogen immediately, washed three times with mannitol, and transferred into Eppendorf tubes with 1 mL of methanol. Next, 400 µL of water containing 1.8 µg/mL methionine sulfone was added and the tubes were vortexed for 30 s. After standing for 1 min, the samples were centrifuged at $187300 \times g$ for 10 min. The upper layer of each sample was retained and freeze-dried. The freeze-dried samples were dissolved in 50 µL of Milli-Q water containing 200 mM of ammonium acetate, 2% formic acid, and 2.5 μ g/mL of phenylalanine- d_5 (internal standard) for CE-time-of-flight (TOF)-MS analysis.

CE-TOF-MS instrumentation

An Agilent CE-TOF-MS (G7100A CE-G6224A TOF/ MS, Agilent, Santa Clara, CA, USA) was used to analyze all samples in cation mode. A fused-silica capillary (80 cm \times 50 μm i.d.) was purchased from Human Metabolome Technologies, Inc. (Woko, Japan) and equilibrated with the BGE (1 M formic acid in water). The temperatures of the capillary and sample tray were set at 20 °C and 5 °C, respectively. The CE-TOF-MS coupling was realized using a coaxial sheath liquid interface containing methanol/water (1:1, v/v) and 0.1 µM hexakis (2,2-difluoroethoxy) phosphazene at 10 μ L/min, which was delivered by an Agilent 1100 series pump equipped with a 1:100 splitter. For traditional analysis, the samples were injected at a pressure of 5000 Pa for 3 s. For multisegmented injection, segments were injected at a pressure of 10 mPa for 40 s and the segments were separated spatially

by BGE zones. The samples were analyzed at 27 kV. The fragmentor voltage, skimmer voltage, and octapole radio frequency voltage were set at 108, 50, and 625 V, respectively. Ionization was performed in positive mode at 4000 V, the drying gas temperature was at 300 °C, and the drying gas flow rate was 7 L/min. The TOF detector was set to scan the mass range m/z 60–1000 at a rate of 1.5 spectra/s.

Method validation

In the sample preparation, $1.8 \,\mu\text{g/mL}$ methionine sulfone was added as internal standard (IS1), which was used to monitor the whole analysis and evaluate the extraction efficiency. Stock solutions of 16 amino acids were freshly prepared before the experiments. The quantification was performed by external calibration. To construct a standard curve, individual stock solutions were serially diluted 2, 5, 20, 50, 100, 500, 1000, 2000, 5000, and 10,000 times with water containing 200 mM ammonium acetate, 2% formic acid, and 2.5 μ g/mL phenylalanine- d_5 (internal standard 2, IS2). The peak area ratios of the analyte to the internal standard area were plotted against the corresponding standard concentrations. To assess the intraday precision and recovery, six replicates were prepared at low, moderate, and high concentrations and analyzed on the same day. The recovery of analytes was approximatively calculated as (measured concentration – endogenous concentration) \times 100/added concentration. To validate the inter-day precision, samples were analyzed each day for three consecutive days.

Results and discussion

Optimization of online sample concentration by multiple injection

Sixteen amino acids (0.75 µg/mL) were used during optimization process, which were listed in Table 1. The optimization effect in Fig. 2-5 is taking Cys as an example. The analytes were dissolved in an aqueous buffer with a high salt concentration and high pH value. Multisegment injection based on hydrodynamic injection of multiple segments of sample that are spatially positioned between BGE in low pH (i.e., space buffer). In this case, The BGE space prevents oxidation of reduced thiols with the sample plug at the anode upon voltage application in CE (D'Agostino et al. 2011). The mobilities of the analytes changed at the pH junction between the analyte solution and the BGE, which resulted in stacking of the analytes at the interface prior to voltage application and the zonal separation was occurred when using an acidic BGE under positive ion mode in Fig. 1. The effects of the salt concentration in the sample solution, the formic acid concentration, and the injection duration on the pH junction were systematically investigated and optimized. For the traditional injection method, one experimental point was optimized at a time. Because the multisegmented injection was performed by hydrodynamic injection of five sample segments that were spatially separated by BGE zones, five experimental points could be optimized simultaneously with the one injection, which dramatically reduced the experimental time.

Effect of salt concentration in the sample matrix

The pH gradient in the capillary was one of the most important experimental variables influencing the sensitivity and resolution of analysis because of its influence on the ionization and mobility of analytes. A pH-mediated gradient was generated by the pH difference between the sample matrix and the BGE. An aqueous ammonium acetate solution was used in the sample matrix to provide a higher pH environment than in the BGE to maximize mobility changes of the metabolites. The effect of varying the ammonium acetate concentration from 0 to 400 mM on the pH gradient was investigated in Fig. 2a. At low ammonium acetate concentrations, the stacking efficiency was not good because there was no difference between the pH of the sample matrix and the BGE. With increases in the ammonium acetate concentration, sample accumulation in the sample zone improved. However, with further increases in the ammonium acetate concentration, the response decreased. In this case, the migration velocity of the pH boundary slowed because of untimely titration of the sample zone.

Effect of formic acid concentration

Formic acid was chosen as the BGE because of its very low pH, which aided formation of a pH environment that was distinct from that of the sample and prevent analyte adsorption. Furthermore, formic acid is a volatile substance that is suitable for MS detection. We tested different formic acid volume fractions ranging from 0.1 to 4% in Fig. 2b. With increases in the formic acid volume fraction, the sample stacking improved but the capillary current increased after voltage application. For the MS detection, the capillary current was not higher than 50 μ A to limit interference with the ESI spray voltage. Finally, 2% formic acid was selected as optimum BGE.

Effect of injection duration

The online preconcentration method allowed for large sample injections because of band narrowing of dilute sample segments within the original sample plug after voltage application to the capillary. Increasing the injection duration can increase the amount of sample in the system and improve the detection sensitivity. In this study, injection durations between 5 and 60 s were investigated in Fig. 2c. With a short injection duration, the

Compounds	Liner range (µg/mL)	R ²	LOD (ng/mL)	Recovery (%	, n=5)		Precision (%)		Standards EF	U251 cell
				1.5 µg/mL	0.75 µg/mL	0.3 µg/mL	Intra-day (<i>n</i> =5)	Inter-day $(n=5,D=3)$		Concentration (µg/mL)
SAH	0.025-5	0.998	5.2	96.9	89.4	93.7	10.7	10.4	32.4	I
ACC	0.0025-2.5	0.997	1.4	76.9	104.3	103.2	3.4	5.6	20.6	I
Ser	0.05-12.5	0.984	25.0	104.5	95.3	113.3	4.7	8.6	22.0	0.2
Cystathionine	0.0125-1.25	0.998	11.7	96.3	89.8	97.3	3.6	10.0	26.0	0.02
Val	0.03-3	1.000	7.5	107.7	103.8	114.5	1.8	4.3	26.9	0.1
Asn	0.03-3	0.996	7.5	103.7	94.7	111.1	2.4	8.6	19.5	0.04
Glu	0.0125-12.5	0.998	6.2	101.5	93.6	111.1	5.0	6.4	25.6	0.1
Cystine	0.025-12.5	0.991	15.1	100.2	97.2	93.0	3.6	8.0	24.3	I
Cys	0.0125-5	0.999	5.0	99.4	93.7	95.7	3.5	6.1	33.1	0.1
Asp	0.025-12.5	0.993	6.9	109.0	94.8	103.5	6.1	12.4	24.4	I
Met	0.0125-5	0.991	5.0	99.2	95.7	96.7	2.8	5.3	27.8	0.1
GSH	0.0125-2.5	0.998	7.0	96.8	94.1	104.9	5.2	18.8	18.1	0.04
S-Carboxymethylcysteine	0.025-12.5	0.993	17.5	100.2	89.0	98.9	5.4	14.3	14.4	Ι
S-Lactoylglutathione	0.0125-5	0.999	1.7	98.3	95.3	101.1	5.8	16.2	17.5	I
Gama-Glu-Cys	0.25-12.5	0.994	203.9	99.2	91.7	105.2	7.1	6.8	18.1	I
Homo-cys	0.0125-5	0.999	8.3	101.2	93.9	88.1	2.8	5.7	25.0	I
EF enrich factor										

Table. 1 Method validation and enrichment effect of the developed method







sample stacking was not efficient. Optimum stacking was obtained with an injection duration of 40 s. With further increases in the injection duration, the online sample preconcentration effect decreased because the large sample volume did not narrow further with longer injection duration. An injection duration of 60 s was not sufficient for further sensitivity improvements. Therefore, 40 s was selected as the optimum injection duration.

Optimization of injection intervals and numbers of segments

Injection intervals of 10 s, 40 s, and 50 s were examined in Fig. 3. The spacing between injections influences the peak capacity because close spacing results in poor resolution of analytes between adjacent sample segments, whereas longer injection intervals prolong the analysis time. As the number of sample segments increased, the space between the sample segments increased. The CE separation efficiency decreased because eluting analytes overlapped with adjacent sample segments in Fig. 4a–c. Larger segment numbers resulted in qualitative and quantitative errors. Therefore, an injection interval of 40 s and five sample segments were selected. Compared with the traditional injection method, multisegment injection improved the sample throughput five times and reduced the analysis time to 4 min for 16 intermediates of sulfur metabolism.

Method validation

Sensitivity of detection

Under the optimized conditions, the calibration curve equation, linear range, correlation coefficient, recovery, and precision were determined (Table 1). The coefficients of determination (R^2) were all greater than 0.984 and the LOD ranged from 1.4 to 203.9 ng/mL. The peak intensity for Cys with the multisegment injection was more

than 33-times that obtained with the traditional injection method in Fig. 5. The sensitivity enhancement factor was calculated as described in a previous study (Kim et al. 2003). The sensitivity enhancement factors (SEFs) for 16











intermediates of sulfur metabolism were listed in Table 1 ranging from 14.4 to 33.1-fold.

Recovery and precision

The recovery of the developed method was evaluated at low, moderate, and high concentrations of 0.3, 0.75, and 1.5 µg/mL. The RSD ranges for the inter-day (n=5) and intra-day (n=5, 3 days) precision in Table 1 were 1.8–10.7% and 4.3–18.8%, respectively. The recovery ranges were 88.1–114.5%, 89.0–104.3%, and 76.9–104.5% at the low, moderate, and high concentrations, respectively.

Application

This method was applied to the analysis of human glioma cell line U-251. The traditional sample analysis method could only detect one metabolite (glutamic acid, Glu) and few signals were observed in Fig. 6. By contrast, seven intermediates of sulfur metabolism—serine (Ser), methionine (Met), GSH, asparagine (Asn), valine (Val), cystathionine, and Cystine—were detected by multisegment injection in Fig. 7. The concentrations of these metabolites were then measured in real cell samples (Table 1). Compared with traditional analysis, the sample throughput was improved five times, and the analysis time was reduced to 4 min for the 16 intermediates of sulfur metabolism with multisegment injection.

Conclusion

Compared with the traditional method, the developed multisegment injection method dramatically reduced the experimental time because five experimental points were optimized simultaneously. With the multisegment injection, the analytical time for 16 compounds in sulfur metabolism was reduced to 4 min/sample, the method throughput was enhanced five times, and the sensitivity was increased 14.4–33.1 times. These results indicate that the developed method will be suitable for biological, clinical, and pharmacological analyses of sulfur metabolism with high sensitivity, throughput, speed, and accuracy.

Abbreviations

ACC: 1-Aminocyclopropanecarboxylic acid; Asp: Aspartic acid; Asn: Asparagine; BGE: Background electrolyte; CE: Capillary electrophoresis; Cys: Cysteine; Glu: Glutamic acid; GSH: y-Glutamyl-cysteine; HPLC: High-performance liquid chromatography; LC–MS: Liquid chromatography–mass spectrometry; LOD: Limit of detection; Met: Methionine; NMR: Nuclear magnetic resonance spectroscopy; RSD: Relative standard deviation; SAH: S-Carboxymethylcysteine; SEF: Sensitivity enhancement factors; Ser: Serine; Val: Valine.

Acknowledgements

We thank Gabrielle David, PhD, from Liwen Bianji, Edanz Editing China (www. liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

Authors' contributions

All authors have equal contribution to this research work. All authors read and approved the final manuscript.

Funding

This work was supported by the Key Research and Development Program of Hebei Province under Grant 20373902D, the Natural Science Foundation of Hebei Province under Grant 82019415024, the National Natural Science Foundation of China under Grant 31800328, the Xi'an City Weiyang District Science and Technology Project under Grant 201940, and the Science and Technology Project of Hebei Province university under Grant QN2020142, the Talent Training Programs of Hebei Province (A201901069), the Scientific Research Key Projects of Education Department of Hebei Province (ZD2019024), the

Scientific Research Key Projects of Education Department of Hebei Province (ZD2021038) and the Scientific Research Projects of Education Department of Hebei Province (Z2020101).

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests

The authors declare that they have no competing interest.

Author details

¹Department of Environmental Engineering, Hebei University of Environmental Engineering, Qinhuangdao, China. ²College of Food and Biological Engineering, Shaanxi University of Science and Technology, Shaanxi, China.

Received: 9 June 2021 Accepted: 17 September 2021 Published online: 28 September 2021

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