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Analysis of amino acids in human blood using UHPLC-MS/MS: Potential interferences of storage time and vacutainer tube in pre-analytical procedure

Tianyi Xia [1], Shouhong Gao [1], Chang Shu, Yan Wen, Yunlei Yun, Xia Tao, Wansheng Chen *, Feng Zhang *

Department of Pharmacy, Changzheng Hospital, Second Military Medical University, Shanghai 200003, PR China

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ABSTRACT

Objectives: To investigate potential interferences of two pre-analytical variables, the storage time and the vacutainer tube, on the quantification of 20 amino acids using a UHPLC-MS/MS method.

Design and methods: Blood samples from 25 apparently healthy subjects were collected into duplicate sets of EDTA-2K, EDTA-3K, coagulation, heparin and citrate tubes, and stored in capped vacutainer tubes at 4 °C for 6 h, 12 h and 24 h before sample analysis. A UHPLC-MS/MS method was established for simultaneous quantification of 20 amino acids. ANOVA for repeated measurement was conducted based on the model of Mauchly's test of Sphericity. Student's *t*-test was applied for comparison between amino acid concentrations obtained from different vacutainer tubes, and consistency of the results was checked through Bland-Altman difference plots and Passing-Bablok regression analysis.

Results: Most of the 20 amino acids showed a least concentration fluctuation with storage time in heparin plasma, followed by EDTA-3K and citrate plasma. The amino acid concentrations were significantly lower in citrate plasma and slightly higher in serum, compared with those in heparin plasma. No fixed bias was observed for amino acid concentrations in EDTA and heparin plasma, but the differences were mostly of statistical significance. Amino acid concentrations in EDTA-3K plasma achieved a good consistency with those in heparin plasma by UHPLC-MS/MS analysis.

Conclusions: Storage time and vacutainer tube were important variables for amino acid analysis. They should draw researchers' attention and then be controlled in good laboratory practice to reduce pre-analytical errors.

1. Introduction

Amino acids (AAs) are recognized as an important class of cellular metabolites in the human body [1]. In addition to acting as the basic units of proteins, they are also involved in a series of biochemical processes like signal transduction, regulation of gene expression and critical metabolic pathways as well as synthesis of hormones and neurotransmitters [2–3]. Metabolomics researches revealed the diagnostic value of the profiling of AAs for certain diseases, because the changes of AA concentrations could ultimately reflect histologic and pathophysiologic alterations and were detectable before the alterations occur [4]. Therefore, AAs were examined as potential biomarkers for the early detection, diagnosis and intervention of some diseases. For example, a selected AA biomarker panel (histidine, isoleucine, valine, and proline) showed a better AUC value in ROC curve analysis for endometrial cancer detection, compared to that of serum CA125 levels (a tumor marker) [5]. Similarly, the urinary D−/L-serine ratio turned out to be a more sensitive indicator of renal ischemia than kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) in the urine, and was more sensitive than creatinine and cystatin C for detecting early renal impairment [6].

By virtue of their potential role in clinical decision making, analysis of blood AAs requires precise quantification results, which however can be influenced by analytical and pre-analytical laboratory errors. In recent decades, remarkable advances in instrument technology, automation and computer science have greatly reduced pure analytical errors [7]. Hence, pre-analytical errors, another source of laboratory

Abbreviations: AA, amino acid; KIM-1, kidney injury molecule-1; NGAL, neutrophil gelatinase-associated lipocalin; Ala, alanine; Met, methionine; Trp, tryptophan; Ile, isoleucine; Leu, leucine; CLSI, Clinical and Laboratory Standards Institute; Ser, serine; Cys, cysteine; Gly, glycine; Thr, threonine; His, histidine; Lys, lysine; Arg, arginine; Val, valine; Tyr, tyrosine; Phe, phenylalanine; Gln, glutamine; Oxo, oxoproline; SDMA, symmetric dimethylarginine; HA, hippuric acid; Kyn, kynurenine; Met-d3, methionine-d3; Ala-d4, alanine-d4; Phe-d5, phenylalanine-d5; PBS, phosphate buffered saline; ESI, electrospray ionization; MRM, multiple reaction monitoring; ISR, incurred sample reanalysis; GLU, glucose; BUN, blood urea nitrogen; SCr, serum creatinine; UA, uric acid; TP, total protein; ALB, albumin; GLOB, globulin; A/G, albumin/globulin; TBIL, total bilirubin; DBIL, direct bilirubin; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, γ-glutamyltransferase; AST, aspartate transaminase; MANOVA, multivariate analysis of variance; ICSH, International Council for Standardization in Haematology; HMDB, Human Metabolome Database.

* Corresponding authors.
E-mail addresses: chenwansheng@smmu.edu.cn (W. Chen), zhangfeng@smmu.edu.cn (F. Zhang).

[1] T.Y. Xia and S.H. Gao contributed equally to this work.

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errors, deserve more attention for result authenticity [8]. The ISO 15189: 2012 standard for laboratory accreditation (United Kingdom Accreditation Service, UKAS) highlighted the necessity to evaluate, monitor and improve all the procedures and processes in the pre-analytical phase, for they were sources of pre-analytical errors that could decisively influence result authenticity [9]. The procedures and processes in the pre-analytical phase include several variables such as sample collection, handling, storage, transportation and preparation for analyses through centrifugation, freezing and thawing, aliquoting and sampling [10]. Among these variables, the vacutainer tube type, which is applied in sample collection, and sample storage time, are two important ones that are vulnerable to be neglected by the analyst or laboratorian [11–14]. Of note, concentrations of some endogenous substances may change, for the identified biochemical reactions during in vitro storage, or be influenced by different vacutainer tubes. The latter is recognized as "tube effects" because tubes can influence clot formation and interact with the components in blood samples [15–18].

It was worth mentioning that explorations into the potential interference of storage time, which had already been conducted on other endogenous substances [16–19], were sparse in AA assays. Moreover, the blood sample was usually not specified of the vacutainer tube type in clinical explorations. Preliminary studies merely focused on the potential interference introduced from vacutainer tube in AA assays, and the interference on some AAs like L-alanine turned out to be inconsistent in several publications [20,21,28].

As measured values for certain blood biomarkers can be influenced by the collecting time point and the blood specimen collection [22,23], efforts must be made to standardize storage and blood sampling procedure in AA analysis. With the prevalence of mass spectrometry due to its high resolution and selectivity, the UHPLC-MS/MS method for quantitative AA analysis proved to be an influential way to investigate the interferences of storage time and vacutainer tube [24–26]. In this study, 25 apparently healthy subjects were recruited to provide blood samples. 20 promising AA biomarkers, from different vacutainer tubes and at different collecting time points, were simultaneously determined by a valid UHPLC-MS/MS method. This study could identify pre-analytical errors from the two variables in AA analysis for better clinical applications.

2. Materials and methods

2.1. Study population

25 healthy subjects without drug-taking, drinking or smoking records from Shanghai Changzheng Hospital were enrolled into the study after writing informed consents. All the subjects had received a physical examination within 3 months before the recruitment, showing no abnormities. Age, gender and biochemical test values were recorded from their medical reports (see Table 1). This study was approved by the Ethical Committee of Shanghai Changzheng Hospital and performed in accordance with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

2.2. Sample collection

Blood collection was carried out at 8:00 a.m. after fasting for 12 h and performed by a single expert phlebotomist, following the instructions of the Clinical and Laboratory Standards Institute (CLSI). The subjects remained a seated position for 15 min before phlebotomy to reduce possible interferences from gesture. Blood samples were drawn using a butterfly connected to a syringe of 20 mL, and then subpackaged into coagulation, citrate, EDTA-2K, EDTA-3K and heparin vacutainer tubes in a fixed order. Parameters of tubes were listed in the Supplementary Materials (Supplementary Table 1). All vacutainer tubes were used according to their respective instructions and before expiration dates. After filling to their capacity, the tubes were gently jolted for 10 times to allow complete mixing of the blood with the anticoagulants or clotting activators of the tube addition. Plasma/serum was then obtained after a clotting period of 30 min at room temperature followed by centrifugation at 4000 × *g* for 10 min. All blood samples were stored for 6 h, 12 h and 24 h in capped vacutainer tubes at 4 °C, and then an aliquote of 50 μL plasma/serum was piped into a 1.5 mL eppendorf tube for analysis.

Table 1
Characteristics of the healthy subjects ($n = 25$).

Item	Concentration
GLU (mM)	4.82 ± 0.29[a]
BUN (mM)	4.53 ± 1.25
SCr (μM)	83.64 ± 14.82
UA (μM)	315.44 ± 95.73
UATP (g/L)	73.84 ± 3.64
ALB (g/L)	48.04 ± 2.28
GLOB (g/L)	25.8 ± 2.63
A/G	1.88 ± 0.21
TBIL (μM)	13.70 (11.00–18.50)[b]
DBIL (μM)	2.60 (2.40–3.15)
ALT (U/L)	16.12 (11.50–18.50)
ALP (U/L)	75.28 ± 18.99
GGT (U/L)	13.00 (9.00–15.00)
AST (U/L)	22.76 ± 5.1

[a] mean ± SD.
[b] median (25th percentile-75th percentile).

2.3. Determination of AAs by UHPLC-MS/MS

2.3.1. Chemicals and reagents

The standards of L-serine (Ser), L-cystine (Cys), L-glycine (Gly), L-threonine (Thr), L-alanine (Ala), L-histidine (His), L-lysine (Lys), L-arginine (Arg), L-valine (Val), L-methionine (Met), L-tyrosine (Tyr), L-isoleucine (Ile), L-leucine (Leu), L-phenylalanine (Phe) and L-tryptophan (Trp) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). L-glutamine (Gln), L-oxoproline (Oxo), L-symmetric dimethylarginine (SDMA), L-hippuric acid (HA) and L-kynurenine (Kyn) were got from Dalian Meilun Biotech Co., Ltd. (Dalian, China). L-methionine-d3 (L-Met-d3), L-alanine-d4 (L-Ala-d4) and L-phenylalanine-d5 (L-Phe-d5, used as the internal standards) were purchased from Toronto Research Chemicals Inc. (North York, Canada). All the standards had a purity >98%. Heptafluorobutyric acid was supplied by Adamas Reagent Co., Ltd. (Switzerland). Phosphate buffered saline (PBS) was got from Shanghai Bio-Light Technology Co., Ltd. (Shanghai, China). Methanol and formic acid were both of HPLC grade. Deionized water was prepared using a Milli-Q Reagent Water System (Millipore, MA, USA).

2.3.2. UHPLC-MS/MS analysis

The AA analysis was performed on an Agilent 1290 series UHPLC system (Agilent Technologies, Germany) coupled to an Agilent 6460 triple-quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Agilent Technologies, Wilmington, DE, USA). An Agilent Zorbax SB-C18 column (3.0 mm × 150 mm, 5 μm) was applied for reverse phase chromatographic separation. The buffer flow rate was 0.4 mL/min with methanol (A) and water (B, containing 0.2% formic acid and 0.02% heptafluorobutyric acid) in gradient elution. The injected sample volume was 2 μL and the preferable column temperature was set at 50 °C. Average system pressure maintained at 64 bar throughout the analysis.

Multiple reaction monitoring (MRM) was selected for data acquisition in the positive mode. The optimized MS parameters were set as follows: drying gas temperature 325 °C, drying gas flow 10 L/min, sheath gas temperature 350 °C, sheath gas flow 12 L/min, capillary voltage 5000 V. Under these conditions, UHPLC-MS/MS analysis was accomplished within 13.5 min.

Method validation was performed according to the FDA recommendations (US Food and Drug Administration, 2013). Linearity regression

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