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A reference measurement procedure for amino acids in blood using isotope dilution ultra-performance liquid chromatography-tandem mass spectrometry

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ABSTRACT

We described a reference measurement procedure for amino acid (AA) quantification in blood samples based on deproteinization with 5-sulfosalicylic acid (SSA) and an isotope dilution-ultra performance liquid chromatography-tandem mass spectrometry (LC–MS) method. The serum was deproteinized with 15% v/v SSA and the supernatant was injected directly into the LC–MS system without further processing. Compared with the use of other precipitants and water as a control, five model AAs—valine, isoleucine, leucine, tyrosine, and phenylalanine—in the SSA-treated samples showed ionization enhancement as well as stable background signals without significant ion suppression effects. Five analytes were clearly separated within 3 min using gradient elution and ion-pair chromatography of water and acetonitrile containing 0.1% v/v trifluoroacetic acid. The limit of detection range of this method was 2–52 fmol, and the RSDs of accuracy and precision from intra- and inter-day assays were within 2.7%. The method was applied to various blood samples including serum, whole blood and plasma, with no reasonable measurement bias revealed. The quantification accuracy of this method was then assessed using commercially available plasma certified reference material (CRM) for AA, and the results agreed well within certified values. We finally applied this method to the determination of candidate serum CRM. The optimized protocol was found to be suitable for the accurate quantification of five AAs in serum, and may satisfactorily serve as a primary method for AA measurement in various blood matrices.

1. Introduction

Metrological traceability is required to produce equivalent test results across method, space, and time in laboratory medicine. It can be achieved through comparison with reference materials and reference measurement procedures [1,2]. For many cases of well-defined clinical measurands, isotope dilution mass spectrometry (ID-MS), which uses isotopic analogues as internal standards, has been used to establish a primary reference measurement procedure [3].

Amino acids (AAs) are important as precursors of products in various biosynthetic pathways and as the primary component of proteins generated in biological systems via protein synthesis, the products of which are subsequently utilized in a variety of metabolic processes [4]. The accurate analysis of AAs in bio-fluids is important in efforts to improve the diagnosis and monitoring of related diseases.

Among the metabolic disorders involving AAs, phenylketonuria (PKU; OMIM 261600) and maple syrup urine disease (MSUD; OMIM 248600) have a high incidence, and early medical intervention has played a large part in treatment strategies. PKU results from a deficiency in the enzyme phenylalanine (Phe) hydroxylase, and is associated with increased Phe levels in blood. Phe hydroxylase is responsible for converting Phe to tyrosine (Tyr), and the Phe/Tyr ratio of PKU patients is higher than that of normal individuals [5]. Increased branched chain AA levels such as valine (Val), leucine (Leu), and isoleucine (Ile) occur in bio-fluid as a result of enzyme deficiency, which has a strong connection with MSUD. Early detection immediately after birth and subsequent implementation of treatment strategies such as the use of specific AA diets are crucial in an attempt to avoid the

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onset of deleterious clinical outcomes [6]. In this study, we select five AAs as analytes, namely Val, Ile, Leu, Phe, and Tyr, which represent biomarkers of certain inherited metabolic diseases.

A variety of methods have been developed for the analysis of AAs, and high performance liquid chromatography (HPLC) related methods have commonly been used due to their selectivity and effectiveness in the analysis of complex samples [7,8]. In particular, the use of hyphenation with MS methods has been extensively developed in the field of amino acid analysis. The excellent selectivity of LC methods can facilitate the separation of AAs with similar structures and isomers within short run times, and MS can identify separated analytes in real time. Moreover, accurate and precise analysis can be realized using isotope analogues as internal standards, referred to as ID-MS as mentioned above [3,8].

Even though the stable isotope analogues as internal standards have the advantages of correcting for sample loss during the process of sample preparation as well as instrument variation, some papers have claimed measurement bias of isotopes induced by matrix [9,10]. The matrix effect is generally defined in LC–MS as the impact that co-eluting compounds endogenously present in the matrix have on ionization efficiency and reproducibility of the ionization source. This phenomenon probably originates from competition in the ion source between co-eluting compounds, and thus the ionization efficacy is very much dependent on the environment of the matrix [11,12]. Since abundant proteins or high molecular weight compounds in blood can affect ionization, appropriate elimination of these molecules is essential for analysis [13–16].

For the diagnosis and/or monitoring of biomarkers, blood is the most commonly-used body fluid because of its ease of collection and diversity of metabolomes present [13]. Depending on its intended use following collection, samples can be utilized as whole blood, plasma, or serum. In certain cases, the sample types and sample pretreatment prior to analysis can affect measurement results, referred to as the matrix effect or matrix bias; therefore, measurement commutability should be demonstrated using different sample types and analytical methods [11,17].

Various methods can be employed for complex biological sample pretreatment such as ultrafiltration, solvent precipitation, solid-phase extraction and liquid–liquid extraction [15,18]. Protein precipitation has been widely employed to remove abundant proteins in blood due to its simplicity. Additionally, organic solvents and organic acids have been commonly employed as precipitants [19–22]. Depending on the target compounds, appropriate precipitants should be carefully considered and examined not only with respect to clean-up yield, but also in terms of analytical selectivity and sensitivity.

In this study, we optimize a deproteinization method of blood for AA analysis and establish an ID-MS method as a primary reference measurement procedure. Deproteinization efficiency, sample loss during preparation and ionization efficiency are also investigated in terms of strict validation to SI unit and compared with other certified reference material (CRM). The optimized method utilizing serum was also applied to plasma and whole blood to assess measurement commutability with respect to sample matrices. Finally, we applied this method to determine a batch of serum certified reference material (CRM) produced by the Korea Research Institute of Standards and Science (KRISS) with an estimation of measurement uncertainty according to the Guide to the Expression of Uncertainty in Measurement (GUM) [23].

2. Materials and methods

2.1. Chemicals

The AA mixture used for calibration contained CRM comprising AAs in 0.1 M hydrochloric acid (HCl) from the National Institute of Standard and Technology (SRM2389a; NIST, Gaithersburg, MD, USA) [24]. The AA mixture was stored at 4 °C before use. The following labeled AAs were obtained from Cambridge Isotopes Laboratory (Andover, MA, USA): L-Valine (U-$^{13}C_5$, 97–99%;^{15}N,97-99%; Val*), L-Isoleucine (U-$^{13}C_6$, 98%; ^{15}N, 98%; Ile*), L-Leucine (U-$^{13}C_6$, 97–98%; ^{15}N, 97–98%; Leu*), L-Phenylalanine (Ring-$^{13}C_6$, 99%; Phe*) and L-Tyrosine ($^{13}C_9$, 99%; Tyr*).

Methanol (MeOH), ethanol (EtOH), acetone, trifluoroacetic acid (TFA), tricholoroacetic acid (TCA), 5-sulfosalicylic acid (SSA) and hydrochloric acid (HCl) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and acetonitrile (ACN) was purchased from Fisher Scientific (Waltham, MA, USA). Water used to prepare the standard solutions, sample solutions and mobile phase was purified using a Millipore Alpha-Q water purification system (Millipore, Billerica, MA, USA), and solvents were filtered through a membrane filter (pore size 0.2 μm) under vacuum. Prior to analysis, all samples were passed through disposable centrifugal filter devices (pore size 0.2 μm, PVDF membrane filter; PALL, Port Washington, NY).

2.2. Blood samples

Various blood matrices were used in this study, specifically whole blood, plasma, and serum.The serum used for method optimization, validation and quantification of AAs was a CRM of frozen human serum, comprising AAs of unknown concentration, from KRISS (CRM No. 111-01-002). The plasma used for method validation and confirmation was a CRM of frozen human plasma with certified values of 12 AAs from NIST (SRM 1950). Both serum and plasma were stored at −20 °C prior to use [25]. Whole blood samples from healthy adults were provided by Chungnam National University Hospital (Daejeon, South Korea); the samples were collected in EDTA tubes and stored at 4 °C prior to use.

2.3. Sample preparation

Blood samples were diluted 10-fold gravimetrically with water. All stock solutions of unlabeled and labeled AAs were also prepared gravimetrically in water. A working internal standard solution was prepared gravimetrically from labeled stocks and concentrations were adjusted to endogenous AA concentrations in the blood samples with preparatory experiments. Unlabeled standard solutions for calibration were prepared to five points in the range of 0.1–100 μmol/kg, with one of the five points set to endogenous concentration. The solutions were stored at 4 °C prior to use.

Three hundred microliters of diluted blood sample and internal standard were placed gravimetrically into a 1.5-mL conical bottom plastic tube and mixed well. An equal volume of 30% *v/v* SSA was added to yield a final concentration of 15%. The tubes were sealed and each solution was mixed prior to being centrifuged at 13,000 rpm for 20 min. Supernatants were filtered using disposable sample filters and then injected into the LC–MS system.

2.4. LC–MS/MS

UPLC–MS/MS analysis was performed using an Acquity UPLC system coupled in-line to a Xevo TQ-S MS system (Waters, Milford, Massachusetts, USA). Chromatographic separation was performed using a CAPCELL CORE ADME analytical column (2.1 mm I.D. x 150 mm, 2.7 μm particle size; Shiseido, Tokyo, Japan) and guard column (2.1 mm I.D. x 5 mm, Shiseido). Mobile phases comprised 0.1% *v/v* TFA in (A) water and (B) acetonitrile with gradient elution at a flow rate of 0.5 mL/min and were utilized as follows: initial conditions A:B = 92:8, linear gradient to 12.5% B for 2.5 min and 30% B for 0.5 min, then switched back to initial conditions and re-equilibration for 1 min. The total run time was 4 min per injection, the sample injection volume was 1 μL and the column oven temperature and sample tray temperature were maintained at 40 °C and 10 °C, respec-