Development of Precolumn Derivatization–LC/MS for Amino-Acid-Focused Metabolomics

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Abstract
Focused metabolomics facilitates the understanding of the current phenotype of living organisms, involves highly practical methods for investigation, and allows for the acquisition of highly quantitative data for important endogenous metabolites. The authors developed novel precolumn derivatization reagents for the analysis of amino acids, which are particularly important endogenous metabolites, through precolumn liquid chromatography/tandem mass spectrometry (LC/MS/MS). One of the reagents facilitated sub-femtomole-to-attomole-scale amino acid detection. We also developed an automated precolumn derivatization amino acid analyzer using another reagent for liquid chromatography/mass spectrometry (LC/MS). With this device, nearly 40 amino acids can be analyzed within a short time (<10 min), compared with the time (~2 h) taken by a conventional automated postcolumn derivatization amino acid analyzer. For highly accurate and precise analysis of free amino acid concentration in human plasma, we validated all steps, from blood collection to measurement, by using the proposed analyzer and developed a test procedure for studying free amino acid metabolomics in human plasma. While calculating the reference intervals of plasma amino acid concentrations in healthy individuals, it was found that the balance of amino acid concentrations in plasma is useful for risk screening of diseases including cancer, diabetes, and myocardial infarction. This functional-group-targeted analysis through LC/MS for amino acid was also effective for dipeptide, tripeptide, and D, L-amino acid analyses and amino acid detection in tissue by imaging mass spectrometry.

Keywords: Focused metabolomics; Precolumn derivatization; Human plasma amino acid; Disease risk screening; LC/MS/MS

1. Introduction
Metabolomics involves the comprehensive quantitative measurement and analysis of endogenous metabolites within a biological system [1-3]. The results of metabolomics-based investigation, along with those obtained from genomics and proteomics, provide essential information for biochemical research. In clinical settings, biomarkers generated through metabolomics-based studies have become one of the most essential diagnostic criteria that can be objectively evaluated as indicators of normal or pathological states [4-6]. Various methods including nuclear magnetic resonance spectroscopy (NMR) [7,8], capillary electrophoresis/mass spectrometry (CE/MS) [9], gas chromatography/mass spectrometry (GC/MS) [10] and high-performance liquid chromatography/mass spectrometry (LC/MS) [11-16] for the comprehensive measurement of endogenous metabolites have been proposed since the beginning of the 21st century. However, it is difficult to analyze a wide variety of endogenous metabolites with significantly different polarities by using only one separation method. Furthermore, it is not preferable to use uniform sample preparation for measurement without paying attention to characteristics such as stability of each endogenous metabolite in a complex system. Inadequate separation and improper sample handling can lead to inaccurate quantification and misinterpretation of results [17,18]. We have developed targeted analysis, using derivatization methods for specific reactions to target functional groups to quantify a selected number of predefined metabolites, which include amino acids [19-25], metabolites with amino groups obtained through specific metabolic pathways [26,27], D-amino acids [28,29], metabolites in the tricarboxylic acid cycle [30], and keto...
acids [31]. An advantage of these methods is the ability to select a suitable sample preparation method for each endogenous metabolite with the same functional group, and to choose an appropriate separation method for each derivative because of the similarity of its physical and chemical properties. This paper describes the development and application of pre-column derivatization reagents specific for amino acids, which are particularly important endogenous metabolites, for LC/MS and liquid chromatography/tandem mass spectrometry (LC/MS/MS).

Free amino acid profiles in blood vary with various disease states. For example, amino acids have been measured in diagnosing inborn errors of metabolism, such as phenylketonuria and for understanding patient malnutrition [32,33]. The Fischer index, which is the ratio of branched-chain amino acids to aromatic amino acids, is a useful indicator for diagnosis and prediction of liver diseases [34].

The first automated chromatographic system for measuring amino acids was developed by Spackman et al. in 1958 [35]. This method adopted a postcolumn derivatization technique, which is based on the colorimetric determination of ninhydrin-derivatized amino acids after separation with a cation exchange resin. Cation-exchange chromatography can effectively separate amino acids [36], and the automated postcolumn derivatization method is excellent in minimizing quantification errors. This method is known as a highly reproducible and robust amino acid quantification method. Until now, the automated analyzer has been conventionally used for assaying not only protein hydrolysates but also amino acids in biological fluids and has been facilitated by the progressive improvement of high-performance liquid chromatography (HPLC) pumping performance and the development of more effective column resins. However, it is difficult to increase the speed of amino acid analysis by cation-exchange chromatography mainly because of the low strength of the resin. The analysis takes about 2 h to measure approximately 40 types of physiological amino acids in a single run. Furthermore, the detection limits are only sub-nanomole range because of colorimetric detection [37].

Since the 1970s, pre-column derivatization techniques have been widely used for amino acid analysis with fluorescence detection [38-40]. Many types of pre-column derivatization reagents, such as o-phthalaldehyde (OPA) [41], 5-(dimethylamino)naphthalene-1-sulfonyl chloride (Dansyl-Cl) [42], 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) [43-45], 9-fluorenylmethyl chloroformate (FMOC-Cl) [46], and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) [47], have been developed for achieving excellent selectivity and sensitivity. The detection sensitivity is within the sub-picomole-to-femtomole range. Amino acid derivatives are more hydrophobic than amino acids; thus, pre-column-derivatization-based HPLC is advantageous in that amino acids can be analyzed by reverse-phase chromatography. In addition, with the recent progress of ultra-high-performance liquid chromatography it has become possible to analyze approximately 20 types of amino acids in about 10 min [40]. However, the separation of amino acid derivatives and the elimination of contaminants, such as reagent hydrolysates, depend on the separation performance of the column. Therefore, as the number of amino acids increases, the time required for separation increases. Short-term analysis of approximately 40 types of amino acids, which are important as endogenous metabolites, has not yet been realized by pre-column-derivatization based HPLC with fluorescence detection. In addition, the method of reacting the sample with the derivatizing reagent before injecting into the column is complicated, which is the reason why this method cannot provide high reproducibility and cannot be easy to use. Therefore, amino acid analysis by pre-column derivatization has not been widely used, except by research experts in analysis.

Mass spectrometry (MS) plays an important role in HPLC metabolite analysis as well as protein analysis. One advantage of LC/MS is its selectivity, which can distinguish analytes not only by their retention times but also by their mass-to-charge ratio (m/z). Furthermore, LC/MS/MS has achieved excellent selectivity and sensitivity, enabling the detection of a specific analyte by using both the precursor ion and the product ion through collision-induced dissociation (CID) and decreasing the noise level, which improves the limits of detection. For amino acid analysis, several methods based on LC/MS and LC/MS/MS have been reported, wherein amino acids in a sample are separated directly by a column without derivatization [48,49]. However, because of the interference of impurities and matrices having the same m/z as that of the amino acid, it is not easy to conduct the quantitative analysis of actual biological samples.

We have recently developed several methods for amino-acid-focused metabolomics by using new types of pre-column derivatization reagents containing the amino group in combination with LC/MS and LC/MS/MS. One of them achieved sub-femtomole-to-attomole-scale amino acid detection. We have also developed a new instrument for the high-speed analysis of amino acid that allows automated pre-column derivatization by using an LC/MS system. The entire analytical process, from blood collection to amino acid analysis by the proposed instrument was optimized, and 21 major types of free amino acids in human plasma were detected, thereby confirming the validity of the analytical method.

Using this developed procedure, we set the reference
intervals for plasma free amino acid concentration in healthy Japanese people and clarified the relationship between plasma amino acid concentration balance and disease risk.

2. Characteristics and application of precolumn amino acid derivatization reagents for LC/MS/MS

A novel precolumn derivatization reagent, p-N,N,N-trimethylammonioanilyl N'-hydroxysuccinimidyld carbamate iodide (TAHS) (Fig. 1), has been developed for the sensitive analysis of amino acids in combination with high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) [19]. The derivatization of an amino acid with TAHS was designed on the basis of the formation of a ureide bond (R-NH-(C=O)-NH-R'), in which R is the component of the amino acid and R' is the reagent moiety. The fragmentation patterns of the derivatives are shown in Fig. 2. The fragment ions from the TAHS adducts were formed because of selective cleavage of the ureide bond between the carbamate moiety and the amino groups and the loss of amino acid moieties from the precursor ions under the optimized positive-ion CID conditions of [M + H]+, m/z 177. The results were utilized to develop the selected reaction monitoring (SRM) conditions, which were programmed to include the transitions of all protonated molecular ions to the common fragment at m/z 177, derived from TAHS (Table 1). All derivatives can be monitored in the positive mode, making it easy to add the monitoring compounds in MS/MS measurements, without any method optimization for product ions. To form the ureide bond with the amino groups of the analytes, we selected an activated carbamate-containing reagent (e.g., succinimidyl carbamate), because carbamates are well-known for their rapid and selective reactions with amino groups under mild conditions such as at 55 °C for 10 min. In addition, as the ionization of the derivatives with TAHS was very efficient because it has a trimethylammonium group, the sensitivity of the system facilitates sub-femtomole-to-attomole-scale amino acid detection by measurement in the SRM mode (Table 1).

Using the precursor ion scan mode of MS/MS, amino acids derivatized with the reagents were simultaneously determined on the chromatogram. This indicated that the method was not only similar to selective amino acid analysis based on the use of fluorescent reagents for the amino groups but also superior to that method, as it includes additional information on the precursor mass number of each peak. Thus, when an unidentified peak is observed on the chromatogram, its structure with the amino group may be estimated from its mass number.

In mass spectrometry studies, reagents labeled with stable isotopes can be used for quantitative comparison of

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**Table 1.** Selected monitor ions and detection limits for each TAHS-derivatized amino acid [19].

| Precursor ion | Product ion | Range (μmol/L) | LOD (S/N=3) (fmol) |
|--------------|-------------|----------------|-------------------|
| Ala          | 177.1       | 0.05-10        | 0.05              |
| Arg          | 177.1       | 0.01-10        | 0.14              |
| Asn          | 177.1       | 0.01-10        | 0.09              |
| Asp          | 177.1       | 0.01-250       | 0.25              |
| Cys2         | 177.1       | 0.025-250      | 0.34              |
| Glu          | 177.1       | 0.025-100      | 0.15              |
| Gly          | 177.1       | 0.01-10        | 0.06              |
| His          | 177.1       | 0.01-10        | 0.16              |
| Ile          | 177.1       | 0.01-25        | 0.1               |
| Leu          | 177.1       | 0.01-10        | 0.1               |
| Lys          | 177.1       | 0.01-50        | 0.07              |
| Met          | 177.1       | 0.01-100       | 0.3               |
| Phe          | 177.1       | 0.01-10        | 0.05              |
| Pro          | 177.1       | 0.01-10        | 0.05              |
| Ser          | 177.1       | 0.01-10        | 0.1               |
| Thr          | 177.1       | 0.01-10        | 0.07              |
| Trp          | 177.1       | 0.01-25        | 0.11              |
| Tyr          | 177.1       | 0.01-25        | 0.06              |
| Val          | 177.1       | 0.01-25        | 0.11              |
samples. We demonstrated that the combination of TAHS and trideuteriummethyl-substituted TAHS, TAHS-d3, was useful in comparing amino acid concentrations between two different samples by using a single LC/MS/MS measurement [19]. We applied this method to several 13C metabolic flux analyses of bacteria involved in amino acid production cultivated with 13C-labeled glucose, measuring intracellular free amino acids [50,51]. In the past, the sensitivity of amino acid analysis was insufficient, so flux analysis was performed using only amino acids in proteins. Since the metabolic turnover of bacteria is fast, it is desirable to use an analytical method such as this method, which has enough sensitivity to directly measure intracellular free amino acids, for accurate metabolic flux analysis. Furthermore, selective cleavage enabled the straightforward isotope ratio analysis of amino acids by the SRM, which was applicable in 13C metabolic flux analysis. When the SRM mode analysis of MS/MS was set at m/z 177, derived from the TAHS moiety of the p-N,N,N-trimethylammonioanilyl group, the precursor ion distribution reflected the isotope ratio of the amino acids.

Although TAHS is an excellent reagent for analysis of amino acids, their enantiomers cannot be separated by a general reversed-phase column. We developed a novel biaryl axially-chiral derivatizing reagent for simultaneous chiral amino acid analysis through LC/MS/MS. (R)-4-nitrophenyl-N-[2′-(diethy lamino)-6,6′-dimethyl-[1,1′-biphenyl]-2-yl] carbamate hydrochloride ((R)-BiAC) was found to be the optimal reagent for highly sensitive simultaneous d, l-amino acid analysis (Fig. 3) [28,29]. Using (R)-BiAC, the complete chiral separation of all derivatized proteinogenic amino acids was achieved within 11.5 min with resolution values of >1.9, except for certain allo-isomers. An exceptional feature of this reagent was its control of elution order, i.e., it afforded the elution of the diastereomers derived from d-amino acids before that of their l-amino acid counterparts for all 19 proteinogenic amino acids. Sensitive detection was also achieved by introducing a dialkyl amino group and selectively cleaving it at the binding site between the reagent and amino acids. Atomolte detection limits were obtained for the tested d, l-amino acids.

Fig. 3. Reaction of amino acid with (R)-BiAC reagent. Dot line is selective cleavage site in MS/MS.

3. Development and validation of LC/MS high-speed amino acid analyzer

In recent years, as the number of samples for amino acid analysis has increased and the samples have become more complex and diverse, it has become necessary to reduce the time for amino acid analysis and improve selectivity. We developed another precolumn derivatized reagent for LC/MS/MS, 3-aminopyridyl-N-hydroxysuccinimidyl carbamate (APDS) (Fig. 4), wherein p-trimethylammonioaniline of TAHS was substituted with 3-aminopyridine. The SRM of ESI-MS/MS in the positive mode was carried out to include the transitions of all protonated molecular ions of the analytes derivatized with APDS to the common fragment at m/z 121, which was derived from the amino pyridyl moiety of the reagent [26].

![Amino acid with APDS reagent](image)

Fig. 4. Reaction of amino acid with APDS reagent.

Although TAHS having quaternary ammonium ions is designed to enhance ionization efficiency and yield sensitive product ions, the separation of the derivatives was not straightforward by reversed-phase HPLC, because of their high hydrophilicity. The retention of the amino acids derivatized with APDS increased compared to those with TAHS in reversed-phase chromatography, because of the higher hydrophobicity of the pyridine moiety in the reagent. Sufficient retention on the column improves the separation of analytes; thus, more than 100 different analytes with amino groups, including amino acids in biological fluids such as mammalian plasma, could be measured within 10 min [26]. Sufficient retention on the column also has desirable effects on the quantitative analysis, because it facilitates the formation of a peak with better shape and prevents ion suppression due to matrix effect.

The novel UF-Amino Station system (Shimadzu Co., Kyoto, Japan), which was configured by combining precolumn derivatization by APDS, high-speed analysis with Shim-pack UF-Amino column (2 μm particle size, 100 mm × 2.1 mm i.d.; Shimadzu Co., Kyoto, Japan), and detection through mass spectrometry with electrospray ionization, was developed for amino acid analysis [52, 53]. The system included LC/MS instead of LC/MS/MS in order to make the device relatively inexpensive and easy to use. Using the UF-Amino Station system, the separation of 38 types of physiological amino acids, including alanine (Ala), α-amino adipic acid (α-AAA), α-amino butyric acid (α-ABA), γ-amino butyric acid (GABA), β-amino isobutyric acid (β-AiBA), anserine (Ans), arginine (Arg), asparagine (Asn), aspartic acid (Asp), carnosine (Car), citrulline (Cit), cystathionine (Cysthi), cysteic acid (CysAc), cystine (Cys2), ethanolamine (EtONH2), glutamic acid
(Glu), glutamine (Gln), glycine (Gly), histidine (His), hydroxy lysine (HyLys), hydroxy proline (HyPro), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), 1-methyl-histidine (1-MetHis), 3-methyl-histidine (3-MetHis), ornithine (Orn), phenylalanine (Phe), proline (Pro), sarcosine (Sar), serine (Ser), taurine (Tau), theanine, threonine (Thr), tryptophan (Trp), tyrosine (Tyr), and valine (Val) which are abundant in foods and biological samples, was achieved in only 8 min (Fig. 5). The interval between injections was 12 min.

Precolumn derivatization procedure is usually performed as manual operations, which makes it difficult to completely control parameters such as reaction time and temperature history. The UF-Amino Station system involved the automated reaction procedure and, therefore, exhibits excellent accuracy and precision. Because the automatic derivatization reaction is carried out individually in parallel with chromatographic separation, the derivatization of the next sample is completed during sample measurement, reducing continuous operation time. In addition, all reagents for the UF-Amino Station system, including APDS and eluents, could be purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan). Preparing a kit of such reagents is also useful as it will reduce the operator effort and time and increase reproducibility of analysis.

The validity of the UF-Amino Station system for measuring human plasma amino acid concentration was evaluated by the detection of 21 major types of free amino acids in human plasma, including Ala, α-ABA, Arg, Asn, Cit, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Orn, Phe, Pro, Ser, Thr, Trp, Tyr, and Val (Table 2) [53]. Limits of detection, lower limit of quantification, linearity, and range sufficiently covered 2.5% to 97.5% of the reference interval of human plasma free amino acid concentration described later. The limits of detection ranged between 0.12 and 2.27 μmol/L, and the lower limit of quantification ranged between 0.39 and 7.55 μmol/L. The limits of detection were five times lower than the lowest limit of linearity for all target amino acids. In the precision evaluation using human plasma, the repeatability was within 4.0%, the intermediate reproducibility was within 6.2%, and the reproducibility was within 7.0%. These results are lower than half of the biological variation of free plasma amino acids in healthy individuals for all amino acids [54]. Recovery rates for spiked plasma samples were between 96% and 103% and the accuracy of twice-diluted plasma samples was between 99% and 104% of the nominal concentration.

These results confirmed that the UF-Amino Station system has sufficient ability to measure the concentration of 21 major types of free amino acids in human plasma and that the system is suitable for use in clinical applications [53]. In comparison with the human plasma free amino acid concentration measured by a conventional amino acid analyzer (postcolumn derivatization method using ninhydrin), the square correlation coefficient was >0.96, except for α-ABA, and the slope ranged between 0.91 and 1.04. The intercept ranged between −5.2 and 11.6. Good accuracy was observed with a good linear correlation. It was shown that this system, with a measurement time of 8 min, can obtain results equivalent to that of an amino acid analyzer using ninhydrin, which takes approximately 2 h for measurement.

4. Proper handling of blood samples for amino acid analysis

Although enormous information can be obtained by analyzing large numbers of endogenous metabolites, the chemical and enzymatic stabilities of most metabolites are unknown. Inappropriate handling of samples that does not consider the stability of each metabolite can lead to inaccurate analytical results and misinterpretations of the results. An advantage of amino-acid-targeted metabolomics is the ability to select a suitable sample preparation method for amino acids with the same functional groups. However, the biochemical and chemical stabilities of each amino acid are different; thus, it is necessary to pay attention to sample preparation even if the targets are limited to amino acids [5,55,56].

Although the concentrations of plasma free amino acids in healthy individuals are under homeostatic control [57], the concentrations are known to show circadian rhythms and some of them vary by 30% within a day [58]. Therefore, it is desirable to collect blood at a fixed time in the day. Because the amino acid concentrations in blood are affected by diet and especially increase after consumption of a high-protein meal [59], blood collection between 7 am and 10 am in a fasting state is desirable. The concentrations of some amino acids are known to be different between blood
Table 2. Linearity, quantification range, limits of detection, lower limits of quantification, and accuracy of major 21 types of amino acids in human plasma [55].

| InternalStandard | Linearity (R) | Range (μmol/L) | LOD (μmol/L) | LLOQ (μmol/L) | Slope | Intercept | Square correlation coefficient | Concentration range (μmol/L) |
|------------------|--------------|----------------|--------------|---------------|--------|-----------|-------------------------------|-----------------------------|
| Ala (3,3,3-d3)   | 0.999        | 25–600         | 2.27         | 7.55          | 0.99   | 11.6      | 0.99                         | 141.7–593.9                |
| α-ABAA 3MeHis (methyl-d3) | 0.998 | 2.5–50         | 0.12         | 0.41          | 0.91   | −2.6      | 0.82                         | 4.5–50.4                   |
| Arg (15N)       | 0.998        | 6.25–250       | 0.62         | 2.06          | 0.95   | 2.8       | 0.99                         | 26.5–124.8                 |
| Asn (13C1,N15)  | 0.999        | 6.25–250       | 0.25         | 0.84          | 0.94   | 2         | 0.99                         | 18.4–251.2                 |
| Cit (4,4,5,5-d3) | 0.999        | 2.5–100        | 0.37         | 1.24          | 0.98   | 0.1       | 0.99                         | 9.3–99.8                   |
| Glu (13C4,N15)  | 0.999        | 2.5–100        | 0.45         | 1.49          | 1.04   | −3.5      | 0.99                         | 9.2–99.9                   |
| Gln (13C4,N15)  | 0.999        | 25–1000        | 1.37         | 4.56          | 0.99   | 6         | 0.96                         | 241.0–998.0                |
| Gly (13C4,N15)  | 0.999        | 25–600         | 0.25         | 0.84          | 1.04   | −5.2      | 0.99                         | 149.4–589.3                |
| His (13C5,N15)  | 0.999        | 12.5–500       | 1.86         | 6.2           | 1.01   | 1         | 0.99                         | 71.3–483.6                 |
| Ile (13C5,N15)  | 0.999        | 12.5–500       | 0.12         | 0.39          | 0.99   | 0.9       | 1                            | 42.7–492.4                 |
| Leu (5,5,5-d3)  | 0.998        | 12.5–500       | 0.5          | 1.67          | 1.01   | 0.4       | 1                            | 85.2–499.6                 |
| Lys (13C4,N15)  | 0.999        | 12.5–500       | 1.54         | 5.14          | 0.97   | 3.6       | 0.99                         | 132.4–493.8                |
| Met (13C4,N15)  | 0.999        | 2.5–100        | 0.14         | 0.46          | 1      | −0.4      | 1                            | 9.1–199.9                  |
| Orn (13C4)      | 0.999        | 6.25–250       | 0.45         | 1.49          | 1.01   | −3.4      | 0.99                         | 19.2–234.0                 |
| Phe (13C4,N15)  | 0.999        | 12.5–500       | 0.52         | 1.73          | 1.01   | 0.9       | 1                            | 46.4–483.8                 |
| Pro (13C4,N15)  | 0.999        | 12.5–500       | 0.73         | 2.44          | 1.02   | −2.7      | 0.99                         | 80.6–489.8                 |
| Ser (13C4,N15)  | 0.999        | 12.5–500       | 1.38         | 4.59          | 1      | 0.3       | 0.99                         | 77.7–482.8                 |
| Thr (13C4)      | 0.999        | 12.5–500       | 0.64         | 2.12          | 0.94   | 8.4       | 1                            | 79.1–496.4                 |
| Trp (13C4,N15)  | 0.999        | 6.25–250       | 0.44         | 1.48          | 1.04   | 1.2       | 1                            | 38.4–252.2                 |
| Tyr (ring 13C8) | 0.999        | 12.5–500       | 1.02         | 3.39          | 0.99   | −0.7      | 0.99                         | 46.0–473.4                 |
| Val (13C5,N15)  | 0.999        | 25–1000        | 0.98         | 3.26          | 0.99   | −0.7      | 0.99                         | 161.3–884.8                |

*Comparison of the amino acid concentrations determined by the UF-Amino Station system and the Hitachi L-8900 amino acid analyzer. A total of 78 types of human plasma samples were used, including 48 samples that were spiked with standard amino acids or were diluted with water to cover the concentration range in the plasma free amino acid reference interval.

Cells and plasma. Although the differences in the concentrations of essential amino acids are small, those of nonessential amino acids can be considerably greater in blood cells [60]. It is important to prevent hemolysis in a sample, and resampling is recommended when severe hemolysis is observed.

If blood samples are left at room temperature after collection, many amino acids undergo reactions because of metabolic enzyme activity from blood cells. For instance, Arg is hydrolyzed to Orn by arginase in blood cells. Gln and Asn are well-known to be metabolized to Glu and Asp, respectively. Gln is also chemically converted to pyroglutamic acid. Amino acids containing sulfur atoms are easily oxidized. It is essential to cool the blood sample to 0 °C immediately after collection to suppress these enzymatic and chemical reactions. With respect to the cooling rate, the blood in the collection tube cools to 0 °C most rapidly in ice water compared with cooling in ice only or in a refrigerator. Blood should not be frozen because it is hemolyzed by freezing [55]. A portable blood tube cooler, such as CubeCooler™, is also useful to cool the blood samples below 0 °C immediately [5]. This cooler is composed of a highly thermally conductive container (aluminum) and an insulator (polyethylene foam), which enable the quick cooling of blood samples, as well as ice water, which maintains the temperature at 0 °C for more than 10 h. Thus, CubeCooler™ may be a useful tool not only for amino acid analysis but also for sample management in other metabolomic studies. It is desirable to separate plasma from the cold-stored blood cells within a few hours. It is important not to contaminate plasma with platelets during centrifugation or plasma sampling. If contamination occurs, the concentrations of some amino acids, such as Asp, Glu, and Tau, are high [55]. Serum is not suitable as a sample for amino acid analysis, because the serum fraction is collected at room temperature for 30 min to remove the coagulation component, and the cellular components and metabolites of platelets are eluted into the serum during the analysis. It is necessary to store the plasma in a −80 °C freezer for long-term storage. When stored at −20 °C, concentrations of some amino acids, especially Arg, Glu, and Cys2, can gradually change [55].

Since plasma contains proteins, such as albumin, deproteinization is necessary before amino acid analysis. When analyzed with an amino acid analyzer through cation-exchange chromatography, plasma is typically mixed with strong acid, such as trichloroacetic acid or sulfosalicylic acid, and is centrifuged. Some amino acids, such as Gln, Asn, and Trp, may be decomposed by acid hydrolysis and it is not possible to accurately quantify these
Table 3) [67]. Reference individuals were selected scientifically evaluated procedure and the method described of plasma free amino acids in Japanese people using the method described. In addition, recovery rates for amino acids depend on the procedure of deproteinization. When analyzing plasma free amino acids with LC/MS or LC/MS/MS, recovery rates can be calculated by adding stable isotope-labeled amino acids as internal standards before deproteinization (Table 2) [53]. Internal standards of stable isotopes are also highly useful in eliminating the influence of matrix effects, which typically limits LC/MS efficiency. Appropriate procedures for blood samples when measuring plasma free amino acids are summarized in Fig. 6.

5. Reference intervals of plasma amino acid concentration in Japanese people

Many studies have reported that plasma free amino acid concentrations are useful for the diagnosis of various diseases, including cancer [61-63], diabetes mellitus [64-66], and malnutrition. However, in those studies, the types of blood samples, pretreatment methods, and analysis methods were different, and their detailed procedure were not described. In addition, it took a long time to analyze the amino acids in each sample. Those were major reasons the fluctuations in free amino acids concentrations in the blood are rarely used in clinical diagnosis except diagnosing inborn errors of metabolism, understanding patient malnutrition, and predicting liver diseases, as mentioned in introduction.

We demonstrated reference intervals for 21 major types of plasma free amino acids in Japanese people using the scientifically evaluated procedure and the method described above (Table 3) [67]. Reference individuals were selected from 7685 subjects examined with the Japanese Ningen Dock (comprehensive health check-up system) in 2008. A total of 1890 individuals (901 males and 989 females) were selected on the basis of exclusion criteria [67], and the reference samples were selected after the outlier samples for each amino acid concentration were excluded. The lower limit of the reference intervals for the 21 types of representative plasma amino acid concentrations was set at the 2.5th percentile and the upper limit at the 97.5th percentile.

By using the nested analysis of variance, we analyzed a large dataset of plasma samples and the effects of background factors (gender, age, and body mass index [BMI]) on the plasma amino acid concentrations. Most amino acid concentrations were related to sex, especially the concentrations of branched-chain amino acids, i.e. Ile, Leu, and Val. Cit, Glu, Orn, and Lys concentrations were related to age. The Glu concentration was also related to BMI [67].

To ensure the reliability of the reference intervals for plasma free amino acid detection, the reliability of the amino acid standard substance and its mixed standard solution used for quantification is important. Although there are some existing products for amino acid standard solutions, those with sufficient accuracy have not been supplied evenly worldwide. The National Institute of Advanced Industrial Science and Technology (AIST/NMIJ) in Japan has developed high-purity certified reference materials for 17 proteinogenic amino acids [68,69] and a method for evaluating the purity of 22 types of non-protein amino acids and the related compounds, which are relevant for clinical chemistry, food chemistry, and pharmaceutical biotechnology [70,71]. In addition, it is necessary to determine the stability of each amino acid present in the mixed solutions in order to prepare metrologically traceable amino acid mixed standard solutions. For instance, the concentration of Glu in 0.1 mol/L hydrochloric acid, which has generally been used for the preparation of amino acid mixed standard solutions, decreased significantly even under cold storage, resulting in its degradation to pyroglutamic acid [72].

Based on the stability studies of each amino acid, the amino acid concentration and solvent pH were optimized, and a certified reference "Amino Acid Mixture Standard Solutions Type H, Type B, and Type AN[CRM]" (FUJIFILM Wako Pure Chemical, Osaka, Japan) for which metrological traceability has been established, has been developed.

6. Disease risk screening by plasma free amino acid profile

Early detection and response to cancer and lifestyle-related diseases are important for extending the
Table 3. Reference intervals of plasma amino acid and concentration of males and females by non-parametric approach [67].

|   | Total Male     | Female       |   | Total Male     | Female       |   | Total Male     | Female       |   |
|---|----------------|--------------|---|----------------|--------------|---|----------------|--------------|---|
|   | Lower limit    | Median       | Upper limit | Lower limit    | Median       | Upper limit | Lower limit    | Median       | Upper limit |
| Ala | 211.6          | 320.1        | 477.2       | 239.1          | 349.7        | 491.8       | 203           | 299.4        | 442.9       |
| α-ABA | 11.2          | 18.8        | 31.6        | 11.7          | 19.6        | 32.5        | 10.7          | 18          | 30.7        |
| Arg | 54.3           | 87.6        | 121.4       | 64.3          | 90.4        | 122.5       | 50.3          | 85.1        | 119.2       |
| Asn | 33.6           | 44.7        | 59.2        | 35.9          | 46.3        | 59.7        | 32.7          | 43          | 58.9        |
| Cit | 18.9           | 29.4        | 42.7        | 28           | 30.2        | 43.3        | 17.7          | 28.6        | 41.7        |
| Glu | 13.5           | 33.5        | 68.9        | 19           | 40.1        | 76.6        | 12.1          | 27.5        | 57.2        |
| Gln | 431            | 562.6       | 691.6       | 467.3         | 576.4       | 702         | 413.8         | 551.5       | 668.6       |
| Gly | 150.2          | 209.4       | 369.6       | 151.3         | 205         | 295.5       | 149.1         | 214.7       | 396.7       |
| His | 63.8           | 79.3        | 97.9        | 66.8          | 82.3        | 101.1       | 62.6          | 76.9        | 92.7        |
| Ile | 36.4           | 55.1        | 85         | 47.4          | 65          | 89.9        | 34.9          | 47.8        | 65.5        |
| Leu | 76.7           | 111.3       | 159.5       | 100.9         | 129.2       | 166.7       | 73.5          | 97.8        | 125.3       |
| Lys | 124.9          | 180.7       | 237.3       | 147.2         | 192.2       | 242.4       | 114.9         | 171.4       | 223.1       |
| Met | 17.8           | 24.6        | 33.3        | 20.2          | 27         | 34.2        | 17           | 22.6        | 29.6        |
| Orn | 27.3           | 48.5        | 72.4        | 36.2          | 52.2        | 75.3        | 24.8          | 45          | 68.4        |
| Phe | 43             | 55.2        | 72.8        | 46.6          | 59.1        | 74.9        | 41.9          | 52.1        | 66.3        |
| Pro | 74.8           | 123         | 221.8       | 94            | 141.7       | 243.3       | 70.4          | 106.2       | 187.9       |
| Ser | 81.5           | 113.3       | 154         | 82.8          | 110.7       | 149         | 80.3          | 115.7       | 157         |
| Thr | 73.7           | 117.3       | 169.3       | 88.3          | 122.6       | 169.6       | 68.1          | 112.4       | 168.5       |
| Trp | 42.9           | 56.3        | 74.4        | 47.4          | 60.4        | 76.9        | 40.9          | 53          | 68         |
| Tyr | 41.9           | 59.3        | 80.9        | 47            | 62.7        | 84          | 39.9          | 56.2        | 74.4        |
| Val | 143           | 205.7        | 287         | 182.1         | 232.9       | 295.3       | 137.5         | 182.7       | 242.5       |

Unit: μmol/L

healthy life expectancy of people in developed countries, especially in Japan. Cancer is the leading cause of death in Japan, and it is extremely important to detect it at an early stage. In terms of lifestyle-related diseases, modern Japan faces the problem of simultaneous overnutrition and undernutrition. It is extremely important to detect the risk of lifestyle-related diseases, such as diabetes, at an early stage, detect protein malnutrition in the elderly, and encourage the review of dietary and exercise habits at the pre-illness stage.

The authors analyzed the concentration fluctuations within the plasma free amino acid profiles of specific patients suffering from cancer and lifestyle-related diseases. Their prodromal symptoms were investigated and a multivariate mathematical model for assessing morbidity risk was developed as an index of multiple plasma amino acid concentrations named “AminoIndex,” which was determined from the vast amount of data obtained by validating all steps from blood sampling to amino acid analysis by the UF-Amino Station system. Specifically, “AminoIndex” can evaluate the current risks of gastric, colon, lung, prostate, breast, uterine/ovarian, and pancreatic cancers. It has also been reported that “AminoIndex” can evaluate the risk of developing diabetes within 4 years. [73-77].

7. Conclusion

The inclusion of a typical derivatization method in HPLC improves detectability and resolution. MS and MS/MS are also useful for improving selectivity and sensitivity. The use of the proposed precolumn derivatization method for LC/MS and LC/MS/MS, which benefits from the advantages of both methods, caused a drastic increase in both the sensitivity and speed of amino acid analysis. The reference intervals of plasma free amino acid concentration in healthy Japanese people were determined through the high-speed precolumn LC/MS amino acid analysis system, UF-Amino Station, after all steps of blood sample management were verified. Since it will now be easier to compare plasma free amino acid concentration fluctuations induced by a disease and the risk thereof with the amino acid reference intervals of healthy people, it is expected that research on the relationship between health and amino acids will accelerate. This review highlights the practical application of the proposed method for disease risk screening by quantifying plasma free amino acids. The analytical method is not only specialized for measuring amino acids in plasma samples but is useful for samples in foods and other biochemical fields [52]. Focused metabolomics by derivatized LC/MS/MS reflects the practical importance of "accurate quantification and analysis."

Precolumn derivatization LC/MS/MS for amino groups can be developed for comprehensive analysis of dipeptides. Dipeptide samples were derivatized with phenyl isocyanate (PIC), and the resulting typical derivatives were analyzed by MS/MS and specific cleavages in CID were observed. These fragmentation patterns have been used for practical analysis combined with the predicted SRM, precursor ion.
scan or constant neutral loss scan [78]. The precolumn derivatization method for LC/MS/MS was also useful for the determination and quantification of tripeptides, such as γ-glutamyl-valyl-glycine that was discovered as a kokumi substance in natural foods [79-84]. Furthermore, the precolumn derivatization method is useful for acquiring semi-quantitative spatial information for multiple amino acids in frozen tissue slices by imaging mass spectrometry. When observing TAHS-sprayed liver tissue sections by matrix-assisted laser desorption/ionization-imaging mass spectrometry, amino acid derivatives can be detected selectively and with high sensitivity [85].

We believe that the proposed method based on the combination of derivatization technology, MS/MS, and LC/MS/MS can be developed and applied in various fields.

Acknowledgments

The authors would like to express their gratitude to the Japanese Society of Clinical Chemistry, the Japanese Society of Amino Acid Sciences, Shimadzu Corporation, Japanese Society of Clinical Chemistry, the Japanese...
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