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Determination of 20 Amino Acids by HPLC/TOF-MS

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To establish an HPLC/TOF-MS method in the determination of human serum amino acids for the ultra-preclinical diagnosis of different diseases. The human serum samples were added inside the isotope-labeled amino acids as the internal standards, then protein-precipitated with CH_3CN and then dried with nitrogen current. After the synchronous derivation, the Diamond RP-C_{18} column was used, with 0.1 % formic acid/water-CH_3CN (0.03 % trifluoroacetic acid) as mobile phase. With the separation and the HR scan mode, the 20 kinds of amino acids and their relative isomers could be qualitatively identified and quantitatively determined inside human serum, which could provide the real time monitoring towards the plasma amino acids spectrum, directly and quickly reflecting the metabolism and nutritional status of the patients with malignant diseases.

Keywords: HPLC/TOF-MS, Amino acid, Simultaneous determination.

INTRODUCTION

The amino acids are the basic units which constitute the proteins, giving the specific molecular structure of the proteins and making the molecules having various biological activities. The dehydration of different amino acids would condense and form peptide (the original fragment of proteins). The generation, existence and extinction of life are all associated with the proteins. The proteins are the important active molecules *in vivo*, if the body lacks the proteins, including the metabolic enzymes and enzyme catalysis, the physiological dysfunction would be resulted in, affecting the body's normal metabolism and leading to diseases. Therefore, the contents detection of amino acids inside the body and in the complex samples, such as serum and urine,would have significant importance towards the clinical diagnosis and scientific research.

Currently, there are many amino-acid-detection technologies. Spackman *et al.*[1] firstly proposed the combination method using the cation-exchange chromatography and post-column ninhydrin-derivation to analyze the amino acids in proteins[1], thus achieving the automatic amino acid analysis[2]. Subsequently, new amino acid analytical methods constantly emerged, pre-column derivation HPLC method[3-5], capillary electrophoresis[6] and mass spectrometry, *etc*. High performance liquid chromatography/electro spray ionization mass spectrometry (HPLC/ESI MS) had been shown to be a useful analytical tool in the quality and quantity determination of the known compounds in traditional Chinese medicine and in *in vivo* small molecules and time-of-flight mass spectrometry (TOF-MS), has been developed for the precise and sensitive analysis. Benefit from the increased resolving power, accurate mass measurement and high full-scan capability, TOF-MS can provide the elemental compositions of the compounds with low accuracy error (routinely within 5 ppm)[7-9]. Currently, this strategy has been successfully developed and applied in the analysis of environmental contaminants including pharmaceuticals and pesticide degradates. However, to the best of our knowledge, only a few researches on the amino acids analysis with this technique have been reported yet.

Based on the previous research and experiences in the quantitative determination of amino acids, in this study, the isotope-labeled internal standards technology was explored for the accurate quantitative analysis of amino acids. The stable isotopes of 5 amino acids were used as the internal standards, and the samples were performed the rapid HPLC-column separation, then the quantitative detection with the TOF-MS. The experiment was simple, the sample pre-treatment was fast, and the data were accurate and reliable, which could provide accurate reference data for the clinical and scientific researches[10-12].

EXPERIMENTAL

The HPLC system was the Agilent 1200 series LC system was employed in this research, which consisted of a G1376A Cap Pump, a G1379B Degasser, a G1365B Multi-Wave Detector, a G1376B Auto sampler and a Hystar PP work station; the TOF-MS was the Brukermicr OTOFQ 125, a hybrid Quadrupole-Time-of-Flight Q-TOF instrument (Bruker Ltd., USA), equipped with an electrospray interface. The electrospray source includes dual nebulizers-one nebulizer for the LC eluent and the other for the internal reference solution. The reference standards was sodium formiate, introduced into the TOF-MS with an automated calibrant delivery system (CDS), which would be used as the internal standard for acute mass weight calibration. Accurate mass measurements of the components were obtained with this calibrant delivery system and thus achieved with this on-line prompt calibration.

The stable isotope-labeled amino acid reference standards were the lyophilized powder (Cambridge Isotope Laboratories), including $^{2}H_3$-leucine, $^{2}H_3$-methionine, $^{13}C_6$-phenylalanine, $^{2}H_3$-aspartate and $^{13}C_6$-tyrosine.

The concentrated stock solution was prepared according to the following procedure: the lyophilized powders of the stable isotope-labeled amino acid reference substances were fully dissolved with 2 mL methanol, and then mixed to form the concentrated working solution, sealed and preserved at 4 °C. In order to ensure the activity of the solution, the concentrated solution should be subpackaged, and discarded after 1 month.

The preparation of the daily working solution was as the following: the concentrated stock solution was diluted 100-fold with methanol, then sealed and preserved at 4 °C. In order to ensure the activity of the solution, the daily working solution should be subpackaged, and discarded after 1 week.

The derivation reagent was prepared (HCl *n*-butanol) as: *n*-butanol-12 mol/L HCl (95:5, v/v), mixed, sealed and preserved at 4 °C.

The resolving solution was the mixture of acetonitrile-water (4:1, v/v), mixed, sealed and preserved at 4 °C.

Collection and preparation of experimental samples: The blood samples were taken from 30 healthy volunteers, with the median age as 40.1 years old. 5 mL whole blood sample was collected in the morning-fasting status and then placed into the heparin-coated tube. The clotting was prevented with shaking the tube upside down 5-6 times; then immediately centrifuged the plasma (within 0.5 h) at 12000 rpm for 10 min; the centrifuged plasma was then transferred to a new EP tube, stored at -20 °C for the further determination. 10 μL sample was added into a 1.5 mL EP tube, with 100 L daily working solution and then centrifuged at 12000 r/min for 2 min. The supernatant was then transferred directly to another 1.5 mL EP tube and dried with nitrogen flow at 40 °C; 60 μL derivative reagent was then added, sealed, vortexed for 30 s, centrifuged instantaneously, then performed the derivation at 65 °C for 15 min. The derivated solution was then centrifuged instantaneously, and dried with nitrogen flow at 50 °C; 100 mL reconstituted solution was then added into, vortexed for 30 s and centrifuged at 12000 r/min for 2 min. 5 μL solution was then injected into the LC/TOF-MS for the analysis.

Chromatographic conditions: The solvents used for HPLC separation were 0.1 % formic acid/water-CH_3CN (0.03 % trifluoroacetic acid) at a flow rate of 1 mL min^{-1}. The mobile phase was as the following: the proportion of B was increased from 10 to 55 % in the first 5 min, then increased to 70 % in 7 min, which was then maintained for 3 min. The column temperature was 55 °C and the Diamond RP-C_{18} column was 100 mm × 2.1 mm, 1.8 mm, with the column temperature as 50 °C and the injection volume as 5 μL.

MS conditions: TOF-MS analysis was performed in positive (ESI+) under the following operation parameters: capillary voltage 4000 V; drying gas 4 L/min; nebulizer 1 psig; gas temp 210 °C; fragmentor voltage 175 V (ESI+); skimmer voltage 60 V; octopole dc1 33.3 V (ESI+); octopole RF 250 V. The full-scan carried out by LC/TOF-MS was recorded across the mass range 50-500 *m/z* to raise the accuracy and sensitivity. Twenty kinds of amino acids for the quantitative analysis were shown in Table-1 and the isotope-labeled amino acids were shown in Table-2.

TABLE-1
IONS OF THE DETECTED AMINO ACIDS IN TOF-MS

Amino acid	Ions in the TOF-MS	
	Calc. MW	Detected MW
Arginine	231.2563	231.2566
Leucine	188.2297	188.2299
Histidine	212.1683	212.1688
Methionine	206.1246	206.1241
γ-Aminobutyric acid	160.0035	160.0032
Phenylalanine	222.1896	222.1891
Serine	162.1195	162.1195
Aspartic acid	246.2299	246.2284
Taurine	126.0209	126.0205
Citrulline	232.2103	232.2112
Proline	172.0776	172.0771
Alanine	146.1892	146.1884
Tyrosine	238.1902	238.1907
Valine	174.2091	174.2084
Hydroxyproline	188.1991	188.1987
Isoleucine	188.2523	188.2529
Glutamic acid	147.1087	147.1081
Ornithine	189.2154	189.2146
Lysine	203.1192	203.1187
Glycine	132.1093	132.1090

TABLE-2
IONS OF THE INTERNAL AMINO ACID STANDARDS IN TOF-MS

Amino acid	Ions in the TOF-MS	
	Calc. MW	Detected MW
$^{2}H_3$-leucine	191.2234	191.2228
$^{2}H_3$-methionine	209.1983	209.1977
$^{13}C_6$-phenylalanine	228.2673	228.2669
$^{2}H_3$-aspartate	249.5692	249.5695
$^{13}C_6$-tyrosine	244.1872	244.1868

Quantitative and data processing: Total ion chromatograph of the samples was processed with the specific TOF-MS acquisition software, then the corresponding EICs (extracted ion chromatograph) was obtained towards the 20 kinds of amino acids for the integration process (Fig. 1). Every 5 μL injected-samples contained 12.5 p mol isotope-labeled internal standards for the calibration with the specific correction

coefficient. After compared the 30 samples of the amino acids results with the stable isotope-labeled internal standards, the specific calibration factor K of each amino acid corresponded to:

$$C_{aa} = A_{aa} \cdot C_{IS} \cdot K / A_{IS}$$

In the equation, the C_{aa} is the amino acid concentration of the test sample; A_{aa} is the amino acid peak area of the test sample; C_{IS} is the amino acid concentration of the internal standard; A_{IS} is the peak area of the internal amino acid standard; K is the specific correction coefficient.

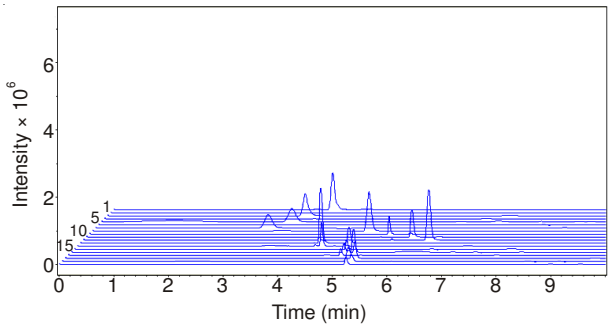

Fig. 1. EIC of the 20 amino acids, 1. Arg; 2. His; 3. Leu; 4. GABA; 5. Phe; 6. Met; 7. Tau; 8. Ser; 9. Val; 10. Ala; 11. Glu; 12. Asp; 13. Cit; 14. Tyr; 15. Pro; 16. Val; 17. Hyp; 18. Gly; 19. Lys; 20. Orn

RESULTS AND DISCUSSION

High-throughput determination method: In this study, the high retention column was used for the separation, and with the help of EIC, specific function of MS, 25 ions were simultaneously detected, involving 20 kinds of amino acids and the 5 corresponding isotope internal standards, which all realized the baseline separation.

Different preparation of biological samples: In this study, different preparation methods were tested, especially towards the elimination of proteins. The centrifugation, CH_3CN deposition and CH_3OH deposition were tested and it was found that no matter the usage of CH_3CN or CH_3OH, the recovery of the method was not satisfactory, which might because the usage of the organic solvent would not only precipitate the protein, but also extract certain amount of amino acids, at the same time. The precipitated proteins would also include certain amount of amino acids, resulting in the loss and the poor recoveries. Therefore, after comparison, the direct centrifugation was used to eliminate the proteins and the result was satisfactory and suitable for the methodology research.

Optimization of TOF-MS parameters: Because the amino acids were acid components, which would exhibited the tail effect in the HPLC column, therefore, certain additives were tested, ammonium acetate, glacial acetic acid, ammonium formate, formic acid and trifluoroacetic acid, *etc*. Different additives would all result in the improvement of the peak shape and retention time, while the trifluoroacetic acid could significantly better the chromatographic parameters, but too much trifluoroacetic acid would inhibit the intensity of the amino acids, therefore the amount of the trifluoroacetic acid should be strictly controlled. The addition of trifluoroacetic acid could provide the H^+, strengthen the signals of the amino acids and increase the noise/signal ration, lower the detection limit.

In order to solve the problem of wide peak, a higher portion of organic reagent was added in, which improved the peak shape and shortened the retention time. At the same time, the oven temperature was set as 55 °C, which could also increase the eluting ability of the mobile phase.

The molecular weights of the amino acids and their derivatives were small, so the small ejection voltage was used, the optimization of the MS parameters was very necessary. Not only the ejection voltage, but also the capillary voltage, drying gas, nebulizer, gas temperature, fragmentor voltage and skimmer voltage should all optimized: too low gas temperature would not evaporate the solvent efficiently, resulting in the inefficient ion generation rate; while too high gas temperature would cause the CH_3CN carbonized in the ejection needle, resulting the blockage of the sample importing. So, after several experiments, the optimized conditions of TOF-MS were established.

Precision: The standard mixture of the internal amino acids as injected into HPLC 6 times continuously and the area of each peak was used for the calculation of precision. The results showed that relative standard deviation (RSD) of peak area of each standard was 5.3, 2.4, 3.6, 4.1 and 3.8 %, respectively, meeting the analysis request of the experiment.

Repeatability: Repeatability was carried out using six samples solution after the same treatment procedure. The results showed that RSD of each peak area was suitable for the analysis requests.

In this study, a rapid and accurate HPLC/TOF-MS method was established, which was further used in the investigation of the clinical significance of amino acids towards the ultra-preclinical diagnosis of multiple blood diseases. The application of the method could be helpful towards the clinical monitoring of the hematologic, providing better health care towards these patients.

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