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Validated Quantitation of Underivatized Amino Acids in Human Blood Samples by Volatile Ion-Pair Reversed-Phase Liquid Chromatography Coupled to Isotope Dilution Tandem Mass Spectrometry

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Quantitation of amino acids in complex matrixes without derivatization is advantageous; however, difficulties exist in both the separation and the detection of those compounds. A validated method that is based on the use of volatile ion-pair liquid chromatography coupled to stable isotope dilution tandem mass spectrometry has been developed for the simple and accurate quantitation of underivatized amino acids in biological samples. Sufficient separation of 22 underivatized amino acids was achieved on a C_{18} column in 36 min using perfluoroheptanoic acid (PFHA) and trifluoroacetic acid (TFA) as mobile phase modifiers. The collisionally activated dissociation spectra of the amino acids were investigated and the transitions of $[M + H]^+ \rightarrow [M + H - 46]^+$, which are specific to α-amino acids, were used for the detection of most amino acids and their stable isotopes. The calibration curves were linear over the range of 0.10−100 μg/mL, and the detection limits were 0.03−20 pmol on column. The quantitative results by this method were compared with those by an established OPA-derivatization HPLC method in the assay of 8 human serum samples, and better recovery and precision data of this method were observed. The method was also applied to the neonatal screening for phenylketonuria (PKU) with dry blood spots, and the results were satisfactory. This is the first time that all proteinogenic amino acids have been quantified directly from biological extracts without any kind of derivatization. The technique shows potential for routine determination of amino acids and analogous compounds in complex matrixes.

Amino acids (AA) are not only the building blocks for the synthesis of peptides and proteins, but also important indicators for some metabolic disorders or physiological processes.[1] For example,[2−5] some free AA in serum are biomarkers for congenital metabolic disorders; ornithine (Orn) and citrulline (Cit) are the biomarkers for the disorder of the urea synthesis cycle. Therefore, it is of great importance to determine the free AA level in biological samples. Quantitation of AA is also valuable for several other fields,[6] such as the food industry and nutrition.

The analytical techniques applied to the analysis of AA have been varied. Since most AA lack chromophore groups and large hydrophobic side chains, the majority of the present methods employ derivatization to allow detection or increase chromatographic separation. The ion-exchange chromatography method for the analysis of AA using postcolumn derivatization was developed in the 1940s.[3,7−8] Various high performance liquid chromatography (HPLC) methods for the analysis of derivatized AA have also been studied since the 1970s .[9−13] Some of these methods have enabled both qualitative and quantitative analysis of most AA. Other analytical methodologies that have been applied to the analysis of AA include gas chromatography[3,8] (GC), thin-layer chromatography[14] (TLC), capillary electrophoresis[15−18] (CE), gas chromatography/mass spectrometry[19,20] (GC/MS), liquid

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chromatography/mass spectrometry[21,22] (LC−MS), tandem mass spectrometry[23] (MS/MS), and capillary electrophoresis/mass spectrometry[24] (CE/MS). Most of the present methods require derivatization (in most cases, either the derivatization procedures were time-consuming or the derivatives were not stable) and laborious sample preparation procedures when assaying biological samples.

Quantitation of AA from a biological matrix *without* derivatization is advantageous: it not only eliminates laborious sample preparation procedures, but also reduces the errors introduced by such problems as derivative instability, side reactions, and reagent interferences. The problems of assaying underivatized AA consist of both the separation and the detection of these compounds. Several methods for the analysis of some underivatized AA by HPLC or CE coupled to electrochemical detectors have been reported.[25,26] With the development of MS/MS technique, high sensitivity and selectivity can be achieved in the assay of complex samples; this opens the possibility that trace amounts of AA can be determined without derivatization. In recent studies,[27,28] mixtures of underivatized AA standards were investigated by LC−MS and CE/MS; however, so far, there is no reliable method that can be employed to simultaneously quantify all proteinogenic AA in very complex matrixes (such as blood samples) without derivatization. This paper proposes a LC−MS/MS method for the quantitation of underivatized AA in biological samples with high speed, sensitivity, and selectivity. This method, which is based on the use of volatile ion-pair HPLC and stable isotope dilution MS/MS, makes it possible for the first time for 22 underivatized AA to be quantified in biological extracts without any further treatment.

Phenylketonuria (PKU, resulting from phenylalanine hydroxylase deficiency) is one of the disorders most widely screened for in newborns,[29] and the neonatal screening for this disease is beneficial, because it provides for early diagnosis and treatment. An accurate method for the screening for PKU, characterized by derivatization of AA with *n*-butanol and quantitation with tandem mass spectrometry, was established by Chace et al.[23] In this report, we performed the method established in this study for the screening of PKU to investigate the feasibility of its application in clinical diagnosis.

EXPERIMENTAL SECTION

Materials. The 22 α-AA were purchased from Sigma Chemical Company (St. Louis, MO), and stable isotopes of those AA (as internal standards) including Gly (D_2, 98%), Ala (D_4, 98%), Val (D_8, 98%), Leu (D_{10}, 98%), Phe (D_8, 98%), Tyr (ring-D_4, 98%), Try (ring-D_4, 98%), Ser (D_3, 98%), Thr ($^{13}C_4$, 98%), Cys (D_2, 98%), Met (D_3, 98%), Asp (D_3, 98%), Glu (D_3, 98%), Asn ($^{15}N_2$, 98%), Gln(D_5, 98%), Arg (D_7, 98%), Lys (D_4, 98%), His ($^{13}C_6$, 98%), Pro (D_7, 98%), Orn (D_6, 98%), Cit (D_2, 98%) were from Cambridge Isotopes (Andover, MA). HPLC grade acetonitrile and methanol were from Tedia Inc. (Fairfield, OH). Formic acid (88%) and trifluoroacetic acid (TFA, 98%) were obtained from Fluka (Buchs, Switzerland), and perfluoroheptanoic acid (PFHA, 97%) was from Acros Oganics (Pittsburgh, PA). Quantitative filter paper (grade 903) for the preparation of blood spots was from Schleicher and Schuell (Keene, NH). **Safety note:** PFHA is a corrosive reagent. Proper safety precautions should be taken to avoid skin/eye contact. Therefore, gloves and goggles should be used when handling PFHA.

Sample Preparation for Serum. Venous blood samples from healthy volunteers were collected between 6:30 am and 7:30 am. The serum layer was carefully collected and processed immediately. A 40-μL portion of serum was transferred to a 1-mL vial, and 160 μL of methanol that contained 10 μg/mL of each internal standard and 0.5% (v/v) formic acid was added to the vial. After vortexing for 10 min and centrifugation at 5000*g* for another 10 min, the supernatant was ready for injection.

Sample Preparation for Dry Blood Spots. Filter paper that contained dry blood spots of neonates collected on the second day postpartum was contributed by the Institute of Inborn Metabolic Disorders of Xinjiang Medical University (Xinjiang Province, China). Among the samples, three were from infants found positive for PKU by bacterial inhibition assay (BIA) and afterward confirmed by other methods; six were from normal infants, and two were from infants who received a false positive by the BIA method. Dry blood spots were punched out from 5.5-mm-diameter circles (equal to 10 μL whole blood) into a 1-mL capped flat-bottomed vial. A 400-μL portion of methanol that contained 0.5% (v/v) formic acid and 1 μg/mL of each internal standard was added to the vial. The extraction was carried out in a 20-watt ultrasonic washer for 15 min. After centrifugation at 5000*g* for 10 min, the supernatant was ready for injection.

LC−MS/MS. A PE SCIEX (Toronto, Canada) API 3000 triple-quadrupole tandem mass spectrometer equipped with a Turbo Ionspray interface, an online degasser, and a Perkin-Elmer binary pump (model 250) was used for LC−MS/MS analysis. For the investigation of the product spectra of AA, 1 μg/mL of the standard solutions were directly infused into the interface by a syringe pump at a rate of 5 μL/min.

For the LC−MS/MS quantitation, separation was carried out on a Dikama Diamonsil C_{18} (particle size, 5 μm; 150 × 2.1 mm) column maintained at 40 °C. The mobile phase consisted of two solvents: 0.8 mM PFHA and 0.05% TFA in water (solvent A, pH = 2.8) and acetonitrile (solvent B). Gradient condition: the proportion of B was 0% for the first 6 min, then increased to 24% in 8 min; 24% was maintained for 7 min, then increased to 60% in 8 min and 60% B was maintained until the end of the analysis. The flow rate was 0.2 mL/min, and the injection volume was 20 μL. The LC effluent was split 3:1 using a valved 3-way split before the interface. Multiple-reaction monitoring (MRM) of MS/MS was used for the specific detection of AA and their stable isotopes. To ensure the sensitivity of detection, we divided those AA into three groups according to their retention times, and only one group of AA was monitored simultaneously during each running period

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Table 1. Products of 22 AA by Collisionally Activated Dissociation

AA	$[M + H]^+$	product ions (sorted by maximum ion intensities, from abundant to weak)[a]
Asp	134	88*, 74, 116, 70, 46, 43
Asn	133	116, 87*, 74
Ser	106	60*, 88, 42
Glu	148	84, 102*, 130, 56, 41
Gln	147	130, 84, 56, 101*, 47
Gly	76	30*, 48
Cys	122	76*, 59, 105, 87
Thr	120	74*, 102, 56, 84, 88, 92
Ala	90	44*
Cit	176	70, 113
Pro	116	70*, 60, 57
Met	150	104*, 133, 61, 56, 102, 74, 87, 84
Val	118	72*, 55
Tyr	182	165, 136*, 91, 119, 95, 77
Ile	132	86*, 69, 104, 57, 44, 41
Leu	132	86*, 104, 55, 44
Phe	166	120*, 103, 77, 93, 149
Trp	205	188, 146, 159*, 132, 118
Orn	133	70, 116, 88, 73, 73
Lys	147	84, 130, 119, 56
Arg	175	70, 116, 130, 158, 60, 133, 88
His	156	110*, 93, 56

[a] An asterisk (*) denotes the product $[M + H - 46]^+$ (immonium ions).

(see Table 2 for detail). The dwell time for each transition was 150 ms, and the pause time for the changes of the scan parameters was 8 ms. The pressure of the collision gas (N_2) was 3.2 mTorr, and corresponding collision energies were applied for each transition. The flow rates of the nebulizer gas (air), curtain gas (N_2), and drying gas (N_2) were 1, 0.6, and 1.2 L/min, respectively. The ion spray voltage, orifice potential, and ring focus voltage were set at 4800, 25, and 160 V, respectively.

Calibration. An internal standard method was used for calibration. Standard solutions of AA were prepared in a solvent consisting of methanol/water/formic acid 80:20:0.1 (v/v) at concentrations of 0.10, 0.50, 2.50, 10.00, 50.00, and 100.00 μg/mL. Two sets of calibration curves were determined for the assay of serum and dry blood spots, and the concentrations of isotopically labeled AA in the standard solutions for those two calibrations were, respectively, 8.0 and 1.0 μg/mL. The data were processed using Macquan software (PE Sciex), and calibration curves were prepared by plotting analyte/internal standard extracting ion current (XIC) peak area ratios vs concentrations. Determination of AA in blood samples was performed using weighted least-squares regression analysis of the standard curves.

Method Validation. The recoveries of AA from serum were determined by standard additions of AA aqueous solutions to a serum sample at three levels (2.0, 8.0, and 40 μg/mL) and then experimentally measuring the added amounts. As for the blood spots, we spiked a whole-blood sample at three levels (0.4, 1.6, and 8.0 μg/mL) and accurately pipetted 10 μL onto filter paper, then cut the entire spot for analysis. Precision of the assay was calculated by repeat analysis of the same blood sample and was estimated as the coefficient of variation (CV%) of the replicate measurements, both intraday and interday.

RESULTS AND DISCUSSION

Volatile Ion-Pair HPLC/Tandem MS/MS. The ability to quantify underivatized AA has been previously limited by the difficulties in separation and detection. To overcome these problems, we used volatile ion-pairing reagents to enable the separation of free AA with a reversed-phase HPLC and MS/MS to enable the detection of underivatized AA. Since the molecular weights of most AA are below 200, the interferences from the mobile phase and the sample matrix are severe for the LC−MS/MS analysis. Therefore, specific MS/MS detection as well as good HPLC separation is necessary for the development of a satisfactory quantitation method.

Since AA are amphoteric compounds, their ionization efficiencies by electrospray ionization (ESI) are relatively low. Therefore,

Table 2. Transitions, Collision Energies, and Periods of Monitoring for MRM of the 22 AA and the Retention Times and Detection Limits by This Method

AA	transitions (AA/ internal standard)	collision energy (eV)	monitoring period (min)	retention time (min)	detection limit (pmol)
Asp	134 → 88/137 → 91	15	0−9	3.1	3
Asn	133 → 116/135 → 118	13	0−9	4.0	2
Ser	106 → 60/109 → 63	15	0−9	4.2	5
Glu	148 → 102/151 → 105	15	0−9	4.5	0.5
Gln	147 → 130/152 → 135	13	0−9	5.4	0.3
Gly	76 → 30/78 → 32	15	0−9	5.8	20
Cys	122 → 76/124 → 78	17	0−9	6.3	15
Thr	120 → 74/122 → 76	15	0−9	6.3	1
Ala	90 → 44/94 → 48	15	0−9	7.4	0.5
Cit	176 → 70/178 → 72	31	9−30	10.9	0.2
Pro	116 → 70/123 → 77	21	9−30	11.4	0.4
Met	150 → 104/153 → 107	15	9−30	16.9	0.08
Val	118 → 72/126 → 80	15	9−30	17.5	0.03
Tyr	182 → 136/186 → 140	19	9−30	18.8	0.06
Ile	132 → 86/142 → 96	13	9−30	24.2	0.07
Leu	132 → 86/142 → 96	13	9−30	25.4	0.07
Phe	166 → 120/174 → 128	17	9−30	26.0	0.04
Trp	205 → 188/209 → 192	19	30−36	31.7	0.2
Orn	133 → 70/139 → 76	21	30−36	33.6	0.2
Lys	147 → 84/151 → 88	19	30−36	34.5	0.4
Arg	175 → 70/182 → 77	29	30−36	34.5	0.6
His	156 → 110/162 → 115	17	30−36	34.6	6

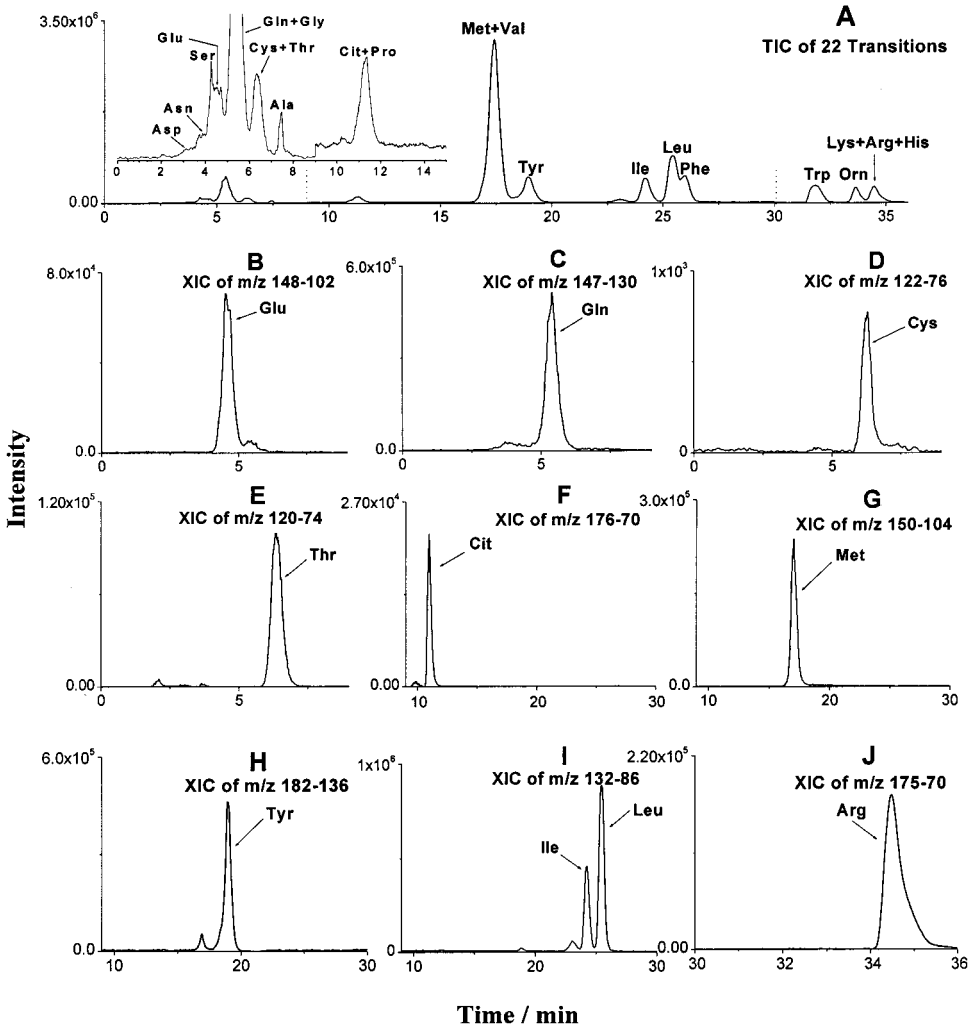
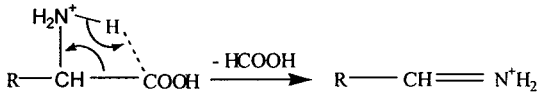


Figure 1. The chromatograms of LC−MS/MS quantitation of 22 AA from human serum: (a) TIC and (b)−(j) nine representative XIC chromatograms of AA. Nine AA and their stable isotopes were monitored simultaneously for the first 9 min, eight AA for the following 21 min, and five AA for the last 6 min.

MS/MS is preferable to MS for the detection of AA because of the higher sensitivity and selectivity it provides. To select a proper transition for the MS/MS detection of an analyte, two factors should be considered: first, the transition should be specific to this compound; second, the intensity of the product ion should be abundant enough for sensitive detection. We investigated the product spectra of the 22 AA under positive ion mode, and the principal CAD products of those AA are shown in Table 1, in which the products of each AA are sorted by their maximum intensities (i.e. the intensity of a product ion acquired under its optimal MS parameters, especially the collision energy), from abundant to weak. Among their CAD fragments, most AA have an abundant product at $[M + H - 46]^+$, which corresponds to the neutral loss of a formic acid by a rearrangement:

Scheme 1



*R stands for the residues of AA

This transition is specific to α-AA, because it involves both the carboxyl and the α-amino group. Therefore, we selected this transition for the detection of most AA. For Asn, Tyr, and Trp, their products $[M + H - 17]^+$ ($-NH_3$) were more abundant than the $[M + H - 46]^+$. However, the former products were not specific enough, so we used the latter for the MRM of those three AA to ensure the selectivity of detection. As to Gln, the product $[M + H - 46]^+$ was too weak to be used for a sensitive detection; therefore, the product at m/z 130 was chosen for MRM. For most basic AA, the products $[M + H - 46]^+$ were very weak or not observed. The authors believe it is because these AA possess extra amino groups whose basicity is stronger than that of the α-aminos, and the places of protonation by eletrospray ionization are more likely at these amino groups rather than the α-aminos; thus, the incidence of the transition $[M + H]^+ \rightarrow [M + H - 46]^+$ is rare. The detection of Arg, Lys, Orn, and Cit was made by employing their characteristic transitions to products at m/z 70, 84, 70, and 70, respectively. The transitions and their corresponding optimal collision energies chosen for MRM of AA are listed in Table 2.

Even with specific MS/MS detection, HPLC separation is evidently needed in this study to eliminate interferences among analytes (for example, the interferences between Leu and Ile, Asn and Asp, Glu and Gln, etc.) and from the sample matrix. Because

Table 3. Quantitative Data for the Determination of 22 AA in Human Serum by the LC−MS/MS Method[a] and by an Established OPA-Derivatized HPLC Method[b,c]

	LC−MS/MS method				established OPA-derivatization HPLC method		
			precision[e] (CV%)			precision[e] (CV%)	
AA	calibration linearity (r^2)	recovery[d] % (SD)	intraday	interday	mean recovery %	intraday	interday
Asp	0.995	93 (3), 96 (2), 98 (4)	2.0	3.6	86	7.3	6.7
Asn	0.997	94 (2), 99 (5), 97 (4)	4.4	6.9	84	8.8	10.3
Ser	0.985	93 (3), 96 (4), 97 (7)	3.9	5.8	91	8.2	9.5
Glu	0.995	104 (3), 97 (2), 99 (6)	2.6	5.9	93	5.7	6.2
Gln	0.990	94 (6), 103 (7), 101 (3)	3.6	8.2	82	4.5	8.8
Gly	0.981	94 (7), 96 (4), 97 (5)	5.8	4.1	91	9.9	10.1
Cys	0.983	92 (3), 95 (4), 97 (3)	2.7	7.5	80	4.7	12.5
Thr	0.996	101 (6), 95 (2), 100 (8)	6.2	5.7	90	5.2	6.7
Ala	0.992	98 (5), 104 (3), 101 (4)	3.0	2.2	86	8.1	7.4
Cit	0.993	96 (5), 98 (7), 105 (11)	3.1	7.6	81	9.4	13.6
Pro	0.999	97 (5), 104 (4), 100 (3)	3.4	5.9	83	7.5	7.2
Met	0.999	105 (7), 103 (6), 102 (3)	3.5	3.7	94	4.2	6.7
Val	0.999	97 (3), 103 (8), 98 (6)	5.7	7.4	90	5.9	7.3
Tyr	0.998	98 (6), 94 (6), 103 (7)	3.4	3.8	87	6.1	6.9
Ile	0.999	98 (8), 102 (5), 97 (5)	4.5	6.3	92	7.1	9.4
Leu	0.997	95 (4), 93 (2), 106 (7)	3.7	5.4	90	5.9	8.3
Phe	0.999	97 (2), 99 (3), 102 (3)	2.6	2.1	91	7.3	6.1
Trp	0.995	92 (5), 96 (8), 95 (6)	5.3	4.9	81	13.5	19.9
Orn	0.998	93 (6), 95 (7), 94 (9)	6.1	8.7	79	23.9	32.1
Lys	0.992	94 (5), 93 (7), 96 (9)	6.4	5.8	91	11.0	12.8
Arg	0.989	105 (7), 96 (5), 102 (5)	3.9	5.5	88	17.3	21.4
His	0.994	91 (4), 95 (9), 95 (5)	4.7	6.3	84	13.9	20.4

[a] Calibration, recovery, and precision. [b] Mean recovery and precision. [c] The recovery and precision data of both methods were measured using aliquots of the same serum sample (a healthy subject). [d] The recoveries of both methods were determined in triplicate at the spiked concentrations of 2.0, 8.0, and 40 μg/mL. [e] Aliquots of a blood sample stored at −70 °C were analyzed six consecutive times in 1 day (intraday, $n = 6$) and twice on three different days (interday, $n = 6$).

AA lack large hydrophobic side chains, mobile phase modifier(s) should be used in order to separate AA efficiently using reversed-phase HPLC. In addition, the modifier(s) should be volatile to avoid a compromise of the ionization and a blockage of the interface. We found that the combination of PFHA and TFA as modifiers in an acetonitrile−water gradient effluent was optimal for our purpose. Additional effects of TFA in the mobile phase that were found were an improvement in the peak shapes and an acceleration of the effluent of some AA. The gradient conditions were optimized. Under the optimized HPLC conditions, good LC/MS/MS chromatographic separation was achieved in the assay of the blood sample extract (Figure 1). This became evident in the discrimination of Ile against Leu, Glu against Gln, etc. Retention times and monitoring periods for 22 AA are shown in Table 2.

The detection limits (S/N = 3) for the 22 AA varied greatly (0.03−20 pmol on column; shown in Table 2) by this method. In contrast to common beliefs,[27] the most sensitive AA by this method were some branched-chain AA (Val, Leu, and Ile; 0.03−0.07 pmol on column) and some aromatic AA (Phe and Tyr; 0.04−0.06 pmol on column), rather than the basic AA (0.2−6 pmol on column).

Problems Associated with the Use of PFHA. We observed that the retention times of AA increased slightly when a number of analyses were performed. The retention times of serveral AA increased by ∼1 min after nine consecutive assays, when the quantitation was rejected by Macquan software because of peak migration. This occurred because PFHA accumulated in the column and slowly modified the surface of the stationary phase. To overcome this, after every six chromatographic runs, we flushed the column with 100% acetonitrile for 30 min to remove the accumulated PFHA.

Quantitation of AA in Human Serum. The calibration curves constructed for the assay of AA in human serum showed good linearity over the concentration range of 0.10−100 μg/mL (r^2 = 0.981−0.999, shown in Table 3). The mean recoveries of 22 AA at the three spiked levels ranged from 91 to 105%, and the intra- and interday precision (CV%) ranged from 2 to 6.4% and from 2.1 to 8.7%, respectively (Table 3). The results for lower limit validation are also satisfactory (shown in Table 4). A matrix effect that could severely impair the LC−MS/MS quantitation[30] of biological samples had not been observed in the development of this method. This should be attributed to the use of stable isotopes as internal standards and sufficient HPLC separation.

Eight serum samples collected from healthy people were assayed. To examine the accuracy of this method, the same samples were also assayed by an established OPA-derivatization HPLC method using an automated on-line HPLC system with precolumn derivatization (derivative reagent, *o*-phthaldialdehyed/3-mercaptopropionic acid) and with norvaline as the internal standard. The quantitative data by the OPA dervetization HPLC methods are also presented in Table 3, and the results for the assay of eight healthy subjects' serums by the two methods are shown in Table 5. Most of the AA concentrations by this method were close to but a little higher than those by the OPA-derivatization HPLC method. The mean recovery values for the 22 AA by the OPA-derivatization HPLC method ranged from 79 to 94%, as compared with 91−105% by this method. In addition,

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Table 4. Recovery and Precision for Lower-Limit Validation[a]

AA	recovery %	precision (CV%) intra-day	precision (CV%) inter-day
Asp	92	7	9
Asn	95	5	7
Ser	91	5	6
Glu	101	3	8
Gln	90	7	12
Gly	86	6	5
Cys	90	5	9
Thr	100	4	6
Ala	87	7	10
Cit	96	5	7
Pro	90	8	9
Met	102	3	7
Val	91	5	6
Tyr	94	6	5
Ile	93	4	8
Leu	90	5	6
Phe	92	6	8
Trp	87	8	11
Orn	89	6	9
Lys	86	7	8
Arg	90	5	9
His	88	7	13

[a] 0.1 μg/mL, $n = 8$.

Table 5. Concentrations[a] of 22 AA from the Serum of Eight Healthy Subjects by This Method and by an Established OPA-Derivatization HPLC Method[b]

AA	AA concn: this method[c]	AA concn: OPA-derivatization HPLC method[c]
Asp	1.25 ± 0.27	0.94 ± 0.22
Asn	6.72 ± 1.83	6.81 ± 1.61
Ser	13.52 ± 2.49	12.98 ± 2.71
Glu	12.80 ± 5.37	10.69 ± 4.79
Gln	95.83 ± 26.42	84.10 ± 22.68
Gly	18.56 ± 3.78	17.84 ± 4.37
Cys	1.05 ± 0.17	0.98 ± 0.20
Thr	18.85 ± 3.39	17.11 ± 3.16
Ala	24.96 ± 5.53	25.6 ± 6.41
Cit	5.71 ± 1.78	4.43 ± 2.60
Pro	24.63 ± 6.18	23.26 ± 5.97
Met	1.11 ± 0.14	0.91 ± 0.17
Val	27.21 ± 5.96	26.33 ± 4.78
Tyr	11.36 ± 3.54	9.77 ± 4.81
Ile	10.11 ± 1.38	9.98 ± 1.05
Leu	15.31 ± 2.04	13.25 ± 1.65
Phe	9.28 ± 2.17	7.79 ± 2.56
Trp	13.53 ± 3.79	11.62 ± 4.03
Orn	8.34 ± 3.09	6.11 ± 3.26
Lys	23.95 ± 6.29	22.89 ± 9.37
Arg	14.27 ± 2.45	13.62 ± 2.97
His	12.13 ± 3.65	13.33 ± 6.03

[a] μg /mL. [b] mean ± SD. [c] $n = 8$.

the repeatability (CV%) for the determination of basic AA such as Arg, Trp, His, and Orn by the OPA-derivatization HPLC method were 13.6−32.1%, in contrast to 3.9−6.4% by this method. We believe that it is a consequence of both the instability of the derivatives and the use of only one or two internal standards for the quantitation of the 22 analytes, most of which possess obvious different chemical and physical properties (such as pI value, solubility, etc.) from those of the internal standard. In this LC/MS/MS method, these problems were overcome by the elimination of derivatization procedure and the use of stable isotopic internal standards.

Table 6. Quantitation of Phe and Tyr from Dry Blood Spots Collected from 11 Neonates

sample ID	Phe concn (μg /mL)	Tyr concn (μg /mL)	Phe/Tyr (molar ratio)
	PKU Patients		
1	118	38.4	3.37
2	71.1	17.0	4.58
3	35.8	12.3	3.19
	Normal Infants		
4	7.75	10.7	0.797
5	9.20	14.1	0.716
6	6.44	7.78	0.907
7	5.91	8.78	0.738
8	6.23	9.18	0.744
9	7.84	9.93	0.789
	PKU False Positive by BIA		
10	19.6	23.4	0.919
11	12.1	16.4	0.809

Quantitation of AA from Dry Blood Spots. The calibration curves for the assay of blood spots were linear over the concentration range of 0.10−100 μg/mL, and the mean recoveries for 22 AA at three spiked levels were from 90 to 112%. The interday and intraday precisions of AA were from 3.3 to 9.7% and from 4.8 to 19%, respectively (Data not shown).

Dry blood spot samples collected from the heels of 11 two-day-old neonates were investigated by this method. The results of Phe and Tyr concentrations and the molar ratios of Phe to Tyr in those samples are summarized in Table 6. Samples numbered 1−3 were from infants diagnosed with PKU, and both the concentrations of Phe and the molar ratios of Phe/ Tyr were remarkably higher than those of the normal infants (samples 4−9). Samples 10 and 11 were from infants first diagnosed with PKU using commercial BIA diagnostic kits, but were afterward found to be falsely positive. We found that both Phe and Tyr concentrations of these samples were obviously higher than those of the normal group, whereas the Phe/ Tyr molar ratios were similar to the normal level, indicating no severe Phe → Tyr translation dysfunction (Phe/Tyr ≥ 2.6 was considered PKU positive).[23] Therefore, a false positive for PKU diagnosis caused by solely using Phe concentration as the marker, which is the basis for the BIA method, can be avoided with this method. This method is also superior to the *n*-butanol-derivatization MS/MS method in the elimination of the laborious derivatization procedure.

CONCLUSIONS:

We have presented a reliable, simple, and sensitive method capable of quantifying all underivatized proteinogenic AA in very complex matrixes. Injection was made right after a very simple extraction step, greatly reducing the laborious and time-consuming sample preparation procedures required by most other methods and, hence, the errors introduced by these procedures. High selectivity was achieved by the combination of sufficient HPLC separation and specific MRM for characteristic transitions. The use of stable isotopically labeled internal standards ensured the

accuracy of quantitation and eliminated matrix effect. By comparing with an OPA-derivatization HPLC method, which is one of the most widely applied methods for AA quantitation, this method was demonstrated to be superior to the latter in the better recovery and precision and the elimination of derivatization procedure. This method is valuable for the routine quantitation of AA from biological samples, such as the screening of diseases for which AA are markers and the investigation of AA kinetics and metabolism using stable isotope-labeled AA.

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