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Direct Quantitation of Amino Acids in Human Serum Using a Stepwise-Dilution Strategy and a Mixed-Mode Liquid Chromatography-Tandem Mass Spectrometry Method

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Abstract : A quantitation method for free amino acids in human serum was developed using a stepwise-dilution method and a bimodal cation exchange (CEX)/hydrophilic interaction liquid chromatography (HILIC)-tandem mass spectrometry system equipped with an electrospray ionization source (ESI/MS/MS). This method, which was validated using quality control samples, was optimized for enhanced selectivity and sensitivity. Dithiothreitol (DTT) was used as a reducing agent to prevent the oxidation of a serum sample (50 μL), which was then subjected to stepwise dilution using 3, 30, and 90 volumes of acetonitrile containing 0.1% formic acid. Chromatographic separation was performed on an Imtakt Intrada Amino Acid column (50 mm × 3 mm, 3 μm) in mixed mode packed with CEX and HILIC ligands embedded in the stationary phase. Underivatized free amino acids were eluted and separated within 10 min. As a result of the validation, the precision and accuracy for the inter- and intraday assays were determined as 2.11-11.51% and 92.82-109.40%, respectively. The lowest limit of quantification (LLOQ) was 0.5-4.0 μg/mL and the matrix effect was 80.22-115.93%. The proposed method was successfully applied to the quantitative analysis of free amino acids in human serum.

Keywords : Amino acid, mixed-mode chromatography, quantitation, serum, LC-ESI/MS/MS

Introduction

Amino acids are essential biological compounds that act as building blocks for peptides, proteins, and neurotransmitters and as precursors to hormones and enzyme cofactors, and they also play an important role as indicators for some metabolic disorders or physiological processes [1,2]. In such metabolic disorders, profiling of amino acids can be utilized as a clinical indicator from biological fluids such as plasma, serum, urine, and cerebrospinal fluid. Therefore, an accurate determination of the concentration of amino acids is essential for a correct diagnosis. A variety of methods based on liquid chromatography–mass spectrometry (LC-MS) without derivatization have been used for the analysis of amino acids, using a strong cation exchange column [3,4] or ion-pairing reverse phase [5,6].

Recently, a mixed-mode column composed of bimodal CEX and HILIC in the stationary phase was developed for the direct quantitation of underivatized amino acids [7]. This mixed-mode column technology provides good peak shape and separation by controlling the pH, salt concentration, and organic solvent. In that study, Yoo et al. [7] simultaneously analyzed underivatized amino acids using this mixed-mode column, which was also applied for the quantitation of amino acids in human serum. Even though the concentrations of amino acids were determined

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using a single dilution method in their study, an improved or modified method would be desirable because amino acids exhibit a diverse range of concentrations in biological fluids. In this context, we envisaged that the development of a stepwise-dilution strategy could be effective to this end.

Herein, we developed a simple, sensitive, selective, and reliable analytical method based on a stepwise-dilution strategy and mixed-mode chromatography for the quantitation of underivatized amino acids in human serum. Such stepwise-dilution up to three times (1:3, 1:30, and 1:90) was designed on the basis of the amino acid profiles of normal serum. In addition, the proposed method was validated and successfully applied to the simultaneous quantitative analysis of amino acids. Finally, concentrations of amino acids in normal human serum were suggested.

Experimental

Chemicals and Reagents

Free amino acids such as L-alanine (Ala), L-arginine (Arg), L-asparagine (Asn), L-aspartic acid (Asp), L-cysteine (Cys), L-glutamic acid (Glu), L-glutamine (Gln), L-glycine (Gly), L-histidine (His), L-isoleucine (Ile), L-leucine (Leu), L-lysine (Lys), L-methionine (Met), L-phenylalanine (Phe), L-proline (Pro), L-serine (Ser), L-threonine (Thr), L-tryptophan (Trp), L-tyrosine (Tyr), and L-valine (Val) were purchased from Sigma-Aldrich Co. (St. Louis, USA). Deuterium-labeled internal standards such as L-alanine-2,3,3,3-d_4 (Aal-d_4, 98%), L-arginine-2,3,3,4,4,5,5-d_7·HCl (Arg-d_7, 98%), L-asparagine-2,3,3-d_3·HCl (Asn-d_3, 94%), L-aspartic acid-2,3,3-d_3 (Asp-d_3, 98%), L-glutamic acid-2,3,3,4,4-d_5 (Glu-d_5, 98%), L-glutamine-2,3,3,4,4-d_5 (Gln-d_5, 97%), L-glycine-N,N,1,2,2-d_5 (Gly-d_5, 98%), L-histidine-d_3 (alpha-d_1, imidazole-2,5-d_2)·HCl·H_2O (His-d_3, 98%), L-isoleucine-2,3,4,4,4,5,5,6,6,6-d_{10} (Ile-d_{10}, 98%), L-leucine-5,5,5-d_3 (Leu-d_3, 98%), L-lysine-4,4,5,5-d_4·HCl (Lys-d_4, 98%), L-methionine-methyl-d_3 (Met-d_3, 98%), L-phenyl-d_5-alanine (Phe-d_5, 98%), L-proline-2,3,3,4,4,5,5-d_7 (Pro-d_7, 98%), L-serine-2,3,3-d_3 (Ser-d_3, 98%), L-threonine-2,3-d_2 (Thr-d_2, 98%), L-tryptophan-imidazole-d_5 (Trp-d_5, 98%), L-4-hydroxyphenyl-2,3,5,6-d4-alanine (Tyr-d_4, 98%), and L-valine-2,3,4,4,4,5,5,5-d_8 (Val-d_8, 98%) were purchased from Sigma-Aldrich Co. (St. Louis, USA), Cambridge Isotope Laboratories (Andover, MA, USA), and CND Isotopes (Pointe-Claire, QC, Canada). Distilled water (DW) was purified using a Milli-Q purification system (Millipore, Massachusetts, USA) and high-performance liquid chromatography-grade acetonitrile (ACN) was purchased from Burdick & Jackson (Ulsan, Korea). Analytical-grade formic acid, ammonium formate, dithiothreitol (DTT), and hydrochloric acid (HCl) were purchased also from Sigma-Aldrich Co. (St. Louis, USA).

Preparation of the Standard Solution and Calibration Standards

All stock solutions of labeled and nonlabeled amino acids were prepared in concentrations of 1 mg/mL and 5 mg/mL, respectively. The deuterium-labeled stock solutions that were used as internal standards (ISTDs) were prepared by dilution using ACN including 0.1% formic acid. Nonlabeled stock solutions were diluted for calibration solutions and quality control (QC) samples using a 0.1 N HCl solution. Calibration ranges were determined on the basis of the concentrations in clinical samples for amino acid diseases such as phenylketonuria and maple syrup urine disease. Final eight-point calibration standards at 4, 8, 20, 40, 80, 160, 240, and 400 μg/mL were prepared for Ala and Glu. The calibration curves were prepared at a factor of 0.75 for each of the above ranges for Gly, Gln, Lys, Ser, and Val; 0.5 for Arg, His, Leu, Thr, Trp, Tyr, Phe, and Pro; 0.25 for Asp and Ile; and 0.125 for Asn and Met (except the highest concentration level). All stock solutions were stored at -20°C.

Preparation of Human Serum and QCSamples

Pooled normal human serums treated with Na-EDTA were purchased from Innovative Research (Novi, MI, USA). The serum samples were stored at -70°C and thawed at room temperature prior to analysis. Then, the samples were homogenized by vortex mixing. 50 μL of DTT solution in 90% ACN (including 0.1% formic acid) was added as a reducing agent to the serum samples (50 μL). The resulting samples were then deproteinized by the addition of 100 μL of the ISTDs solution in ACN with 0.1% formic acid. The samples were vortexed and then centrifuged at 12,000 rpm for 10 min at 4°C, and then the supernatant was taken and serially diluted using an ISTDs solution to prepare the 1:30 and 1:90 diluted samples.

Instrumentation

An LC system consisting of an LC-20 AD XR series chromatograph (Shimadzu, Kyoto, Japan) was coupled to an API 4000™ mass spectrometer (AB Sciex, Toronto, Canada) equipped with a TurboIonSpray source. Liquid chromatographic separation was performed at 35°C on a mixed-mode Intrada Amino Acid column (50 mm × 3 mm, 3 μm, Imtakt Co.). The mobile phase consisted of ACN including 0.1% formic acid (A) and 100 mM ammonium formate in DW (B) at a flow rate of 0.5 mL/min. The following gradient program was used: the linear gradient was increased from 10% to 90% B in 7 min, held for 1 min, and returned to the initial conditions in 0.1 min, followed by 2 min of equilibration.The total run time was 10 min, and the injection volume was 1 and 2 μL for 30, 90 volumes and 3 volumes of protein precipitation, respectively.

The mass spectrometric analyses were performed with an ion spray voltage of 5,400 V, turbo gas temperature of 650°C, ion source gases I and II at 80 psi, and curtain gas

at 30 psi. The declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) were optimized by the autotuning procedure of the instrument. The mass spectrometer was used in the multiple reaction-monitoring mode (MRM) in positive mode. Data acquisition and processing and the control of the MS were performed using the Analyst 1.5 software.

Validation Procedure

The developed method was evaluated for linearity, the lowest limit of quantification (LLOQ), intra- and interday accuracy and precision, and matrix effect. The detailed procedures were mainly based on "FDA-Draft Guidance for Industry: Bioanalytical Method Validation (2013)" [8].

The calibration curve was prepared by determining the best fit of the peak area ratio (peak area of analyte/ISTD) versus concentration. The linearity of the calibration curve was evaluated by analyzing amino acid standard solutions at eight concentrations with three replicates, and the resulting correlation coefficients (r^2) were evaluated for linearity. The level of the LLOQ was established as a signal-to-noise ratio of ≥10.

The concentrations of the LLOQ, low QC (LQC), medium QC (MQC), and highQC (HQC) were processed. Five replicates in the same run (intraday accuracy and precision) and three replicates in three separate runs (interday accuracy and precision) were analyzed. Precision was calculated as the %coefficient of variation (%CV) of the assayed concentrations. Accuracy was expressed as the bias of the assayed concentration to the expected value.

Endogenous amino acids were tested with spiked human serum samples at LQC, MQC, and HQC levels (n = 3) in order to evaluate the matrix effect. The LQC, MQC, and HQC spiked samples, blank serum samples, and neat solution samples were analyzed, and the values obtained for the peak area were used for the calculation of the matrix effect as follows: % matrix effect = [(peak area of the QC spiked sample - peak area of the blank sample)/ peak area of the neat solution] × 100.

Results and Discussion

The simultaneous analysis of endogenous multi-target analytes with a diverse range of concentrations constitutes a particular challenge due to the inherent limited dynamic range for quantitation and different ionization efficiency for analytes in the ESI source of the LC-MS/MS technique. We provided that the proposed sample preparation method could overcome this problem via a stepwise-dilution strategy. One serum sample was sequentially diluted to 1:3, 1:30, and 1:90 according to the concentration levels of normal serum and ESI efficiency of amino acids. Thus, the 1:3 dilution was applied for Asn, Asp, and Gly; the 1:30 dilution was applied for Ala, Arg, Glu, Gln, His, Ile,

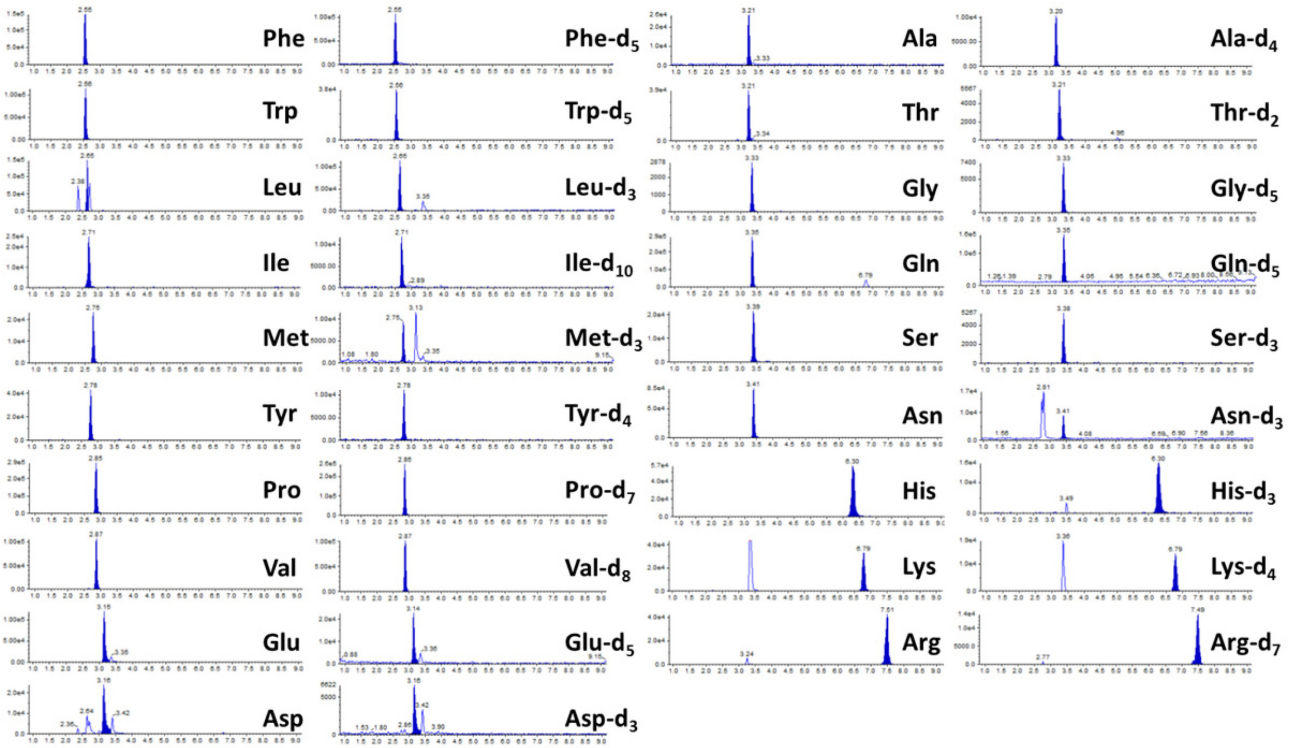

Figure 1. Representative MRM chromatograms for 19 free amino acids and internal standards in normal human serum using LC–ESI/MS/MS with mixed-mode column and three-step dilutions.

Lys, Met, Ser, Thr, Trp, and Tyr; and the 1:90 dilution was applied for Leu, Phe, Pro, and Val. The present sample preparation method could minimize the peak saturation and low intensity of target analytes from the matrix. The dynamic ranges of amino acids for quantitation were optimized according to normal concentration levels and high concentration levels of those amino acids affected by metabolic disorders. Figure 1 shows the MRM chromatograms of the samples subjected to LC–ESI/MS/MS analysis following the present procedure. As can be seen, most amino acids showed a satisfactory peak shape and selectivity under the present liquid chromatographic condition, except for Leu and Ile, which were partially separated. No interference was observed at the retention times of amino acids. In addition, no carry-over effect was found in our system. The conditions of the mixed-mode column, such as the pH, type of organic solvent, acid modifier concentration, and salt concentration, seemed to have an effect on peak retention, separation, and elution. In particular, the salt concentration was found to affect the interaction (elution strength) between the analytes and the stationary phase, and the use of a high salt concentration (100 mM ammonium formate) as a mobile phase required careful washing of the instrument after the analysis of the samples.

Table 1 summarizes the dynamic ranges, linearities, and LLOQs of amino acids. The regression coefficients (r^2) for the calibration curves exceeded 0.99, from which a good

Table 1. Dynamic ranges, linearities (r^2), and LLOQs of 19 free amino acids using LC–ESI/MS/MS.

Comps. (Abbrs.)	Dynamic range	r^2	LLOQ (μg/mL)
Asn	0.5-100	0.9985	0.5
Asp	1-100	0.9972	1.0
Gly	3-300	0.9983	3.0
Ala	4-400	0.9987	4.0
Arg	2-200	0.9961	2.0
Glu	4-400	0.9969	4.0
Gln	3-300	0.9966	3.0
His	2-200	0.9987	2.0
Ile	1-100	0.9939	1.0
Lys	3-300	0.9950	3.0
Met	0.5-100	0.9948	0.5
Ser	3-300	0.9999	3.0
Thr	2-200	0.9982	2.0
Trp	2-200	0.9969	2.0
Tyr	2-200	0.9978	2.0
Leu	2-200	0.9994	2.0
Phe	2-200	0.9992	2.0
Pro	2-200	0.9985	2.0
Val	3-300	0.9983	3.0

Table 2. Validation results for intra- and interday accuracy, precision, and matrix effect of 19 free amino acids using LC– ESI/MS/MS.

Comps. (Abbrs.)	QCs	Conc. (μg/mL)	Intraday(n = 5)		Interday(n = 3)		Matrix effect (%)
			Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	
Asn	LLOQ	0.5	96.92	3.88	99.94	4.05	–
	LQC	1	100.88	5.44	101.80	4.33	110.47
	MQC	40	96.64	5.69	98.47	4.40	91.63
	HQC	80	98.18	3.75	101.17	4.28	93.44
Asp	LLOQ	1	102.26	3.82	98.60	3.62	–
	LQC	2	98.00	4.86	98.80	4.61	98.87
	MQC	40	96.42	7.56	99.27	6.55	105.75
	HQC	80	97.20	3.90	98.66	7.63	106.38
Gly	LLOQ	3	96.16	3.81	101.87	8.71	–
	LQC	6	102.44	6.84	101.63	6.48	103.26
	MQC	120	96.00	3.02	98.69	4.83	98.73
	HQC	240	100.70	9.98	100.30	8.57	94.73
Ala	LLOQ	4	103.74	11.51	97.59	3.56	–
	LQC	8	109.02	6.15	99.90	11.20	99.39
	MQC	160	99.14	7.28	102.62	6.56	98.19
	HQC	320	99.32	8.96	108.01	5.74	93.99
Arg	LLOQ	2	101.08	7.04	100.71	3.46	–
	LQC	4	102.18	7.68	103.17	7.23	112.34
	MQC	80	99.98	4.54	100.93	3.86	91.54
	HQC	160	98.46	3.86	102.24	3.77	90.95
Glu	LLOQ	4	100.06	4.50	99.80	6.42	–
	LQC	8	95.62	3.41	101.51	5.14	113.14
	MQC	160	100.06	5.86	96.70	5.05	87.09
	HQC	320	101.48	6.26	99.07	6.10	91.31

Table 2. Continued.

Comps. (Abbrs.)	QCs	Conc. (μg/mL)	Intraday(n = 5)		Interday(n = 3)		Matrix effect (%)
			Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	
Gln	LLOQ	3	99.72	8.07	96.22	4.08	–
	LQC	6	102.26	8.25	99.74	7.95	106.69
	MQC	120	99.52	4.01	100.28	6.24	89.37
	HQC	240	97.54	5.24	95.79	3.20	96.94
His	LLOQ	2	109.40	4.02	105.89	3.15	–
	LQC	4	104.58	7.62	101.22	6.03	100.56
	MQC	80	105.18	5.97	102.57	6.60	80.22
	HQC	160	104.22	5.37	102.07	6.79	92.42
Ile	LLOQ	1	107.14	10.94	99.29	8.83	–
	LQC	2	103.26	5.79	99.77	10.58	102.54
	MQC	40	105.48	6.19	103.08	4.64	98.07
	HQC	80	96.46	6.34	98.36	5.54	96.01
Lys	LLOQ	3	100.50	2.51	96.72	6.60	–
	LQC	6	99.48	6.16	94.28	6.23	112.95
	MQC	120	97.72	7.36	94.09	4.09	89.62
	HQC	240	104.32	6.29	100.37	6.29	100.54
Met	LLOQ	0.5	96.20	5.60	104.14	9.22	–
	LQC	1	94.28	11.14	100.36	7.59	106.47
	MQC	40	101.42	6.45	99.38	6.10	101.77
	HQC	80	99.88	7.12	96.44	6.23	94.10
Ser	LLOQ	3	108.72	8.54	104.37	8.52	–
	LQC	6	97.34	7.04	97.65	7.03	115.93
	MQC	120	94.06	2.11	97.69	3.56	90.93
	HQC	240	94.72	5.97	100.11	7.22	97.53
Thr	LLOQ	2	108.10	6.79	108.94	5.99	–
	LQC	4	106.76	7.67	102.81	8.31	102.19
	MQC	80	97.28	7.32	98.46	5.52	95.67
	HQC	160	98.78	4.86	97.47	7.43	97.92
Trp	LLOQ	2	103.50	5.95	104.08	6.54	–
	LQC	4	100.72	5.14	98.74	7.21	106.01
	MQC	80	100.36	3.26	101.88	5.78	97.65
	HQC	160	93.34	2.19	100.20	5.68	96.23
Tyr	LLOQ	2	99.82	9.41	96.11	5.65	–
	LQC	4	109.14	5.81	101.99	8.32	105.84
	MQC	80	108.80	5.34	101.47	5.99	96.91
	HQC	160	108.20	4.31	101.91	5.33	97.25
Leu	LLOQ	2	92.82	4.45	97.76	6.53	–
	LQC	4	95.40	3.09	97.39	5.27	97.46
	MQC	80	103.56	4.09	102.30	3.59	93.15
	HQC	160	100.58	4.37	100.52	4.59	96.14
Phe	LLOQ	2	96.14	6.19	100.48	6.65	–
	LQC	4	100.74	5.59	101.89	5.79	106.31
	MQC	80	101.72	7.19	100.03	4.85	91.99
	HQC	160	96.24	3.10	96.50	6.63	93.90
Pro	LLOQ	2	96.14	5.86	95.14	8.13	–
	LQC	4	100.78	3.64	101.54	3.64	94.88
	MQC	80	99.98	3.04	99.88	3.84	91.36
	HQC	160	96.10	3.63	96.87	6.15	97.72
Val	LLOQ	3	101.54	6.78	105.84	5.41	–
	LQC	6	104.24	6.56	102.80	4.17	102.14
	MQC	120	100.78	7.04	98.82	4.43	97.92
	HQC	240	98.50	4.12	94.07	6.77	98.37

Table 3. Results for the determination of the concentrations of 19 free amino acids in normal human serum samples.

Comps. (Abbrs.)	Conc. (μg/mL, mean±SD (n=3))			Total mean conc. (μg/mL)
	Sample 1	Sample 2	Sample 3	
Asn	6.7 (±0.4)	6.6 (±0.3)	6.5 (±0.2)	6.6 (±0.1)
Asp	9.8 (±0.4)	10.5 (±0.4)	10.7 (±0.2)	10.3 (±0.5)
Gly	28.5 (±1.8)	28.9 (±1.5)	29.9 (±1.4)	29.1 (±0.7)
Ala	50.9 (±1.0)	48.0 (±1.7)	47.2 (±0.6)	48.7 (±1.9)
Arg	21.3 (±1.0)	21.3 (±1.0)	23.9 (±0.7)	22.2 (±1.5)
Glu	50.9 (±2.5)	49.5 (±1.3)	54.5 (±1.6)	51.6 (±2.6)
Gln	15.5 (±0.7)	15.1 (±0.8)	15.3 (±0.4)	15.3 (±0.2)
His	13.1 (±0.8)	13.2 (±0.8)	14.4 (±0.8)	13.6 (±0.7)
Ile	11.7 (±0.8)	12.2 (±0.6)	11.3 (±0.5)	11.7 (±0.5)
Lys	32.0 (±1.0)	33.0 (±1.6)	34.3 (±2.0)	33.1 (±1.2)
Met	4.3 (±0.1)	4.8 (±0.2)	5.0 (±0.2)	4.7 (±0.4)
Ser	25.4 (±1.2)	24.4 (±0.5)	26.2 (±1.1)	25.3 (±0.9)
Thr	16.6 (±0.9)	16.0 (±0.8)	17.1 (±0.4)	16.6 (±0.6)
Trp	12.8 (±0.2)	12.5 (±0.5)	12.3 (±0.1)	12.5 (±0.3)
Tyr	12.9 (±0.7)	13.5 (±0.6)	12.5 (±0.5)	13.0 (±0.5)
Leu	25.9 (±1.0)	26.5 (±1.4)	28.0 (±0.5)	26.8 (±1.1)
Phe	24.7 (±0.9)	24.7 (±1.1)	22.0 (±0.2)	23.8 (±1.6)
Pro	22.2 (±0.4)	24.8 (±0.5)	24.4 (±0.7)	23.8 (±1.4)
Val	34.1 (±0.7)	33.9 (±0.7)	33.7 (±1.1)	33.9 (±0.2)

linearity was confirmed. The LLOQs ranged from 0.5 to 4.0 μg/mL. As shown in Table 2, the intra- and interday accuracies ranged from 92.82% to 109.40% and from 94.07% to 108.94%, respectively. The intra- and interday precision values were lower than 11.51% and 11.20%, respectively, for all the concentration levels of all amino acids. The matrix effect, which can significantly affect the ionization of the analyte by causing a reduction of the MS/MS response, was evaluated and established to lie within a range of 80.22% to 115.93%.This indicates that the matrix effects were not significant.

Finally, the present method was applied to the quantitative analysis of human serum samples. Table 3 summarizes the results obtained from the determination of the concentrations of amino acids from normal human serum samples. Samples 1, 2, and 3 were prepared from the same serum batch in order to investigate the repeatability of the stepwise-dilution. Each sample was analyzed in triplicate (n = 1, 2, and 3 in Sample 1) to investigate the reproducibility of the analytical method. As shown in Table 3, the stepwise-dilution sample preparation method showed good repeatability that ranged from 0.1 to 2.6 in the three samples of amino acids. Furthermore, each sample showed good reproducibility, which ranged from 0.1 to 2.5 in triplicate analysis. From these results, we suggested that present method is reliable for the determination of the concentration levels of amino acids in normal human serum.

Conclusions

In this study, we developed an analytical method for the quantitation of amino acids in human serum using a stepwise-dilution and bimodal CEX/HILIC LC–ESI/MS/MS method. The proposed method was validated and successfully applied to the simultaneous quantitative analysis of 19 underivatized free amino acids. The results presented here demonstrated that this method is reliable and reproducible for direct quantitation of free amino acids in human serum.

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