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Short communication

Rapid resolution liquid chromatography (RRLC) analysis of amino acids using pre-column derivatization

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ABSTRACT

A rapid resolution liquid chromatography (RRLC) method was developed for the simultaneous determination of 23 amino acids in rat serum after pre-column derivatization with 2,4-dinitrofluorobenzene (DNFB). The amino acid derivatives were separated on an Agilent Zorbax Eclipse Plus C_{18} (4.6 mm × 50 mm, 1.8 μm) column at 45 °C. Ultraviolet (UV) detection was set at 360 nm. Good separation of 23 amino acids was achieved within 10 min with a ternary gradient elution of mobile phase at a flow rate of 1.5 mL min^{-1}. Calibration curves were linear over the range from 1 to 500 μmol L^{-1} with coefficients 0.9962 or better for each amino acid. The lower limits of quantification (LLOQ) of all 23 amino acids were 1 μmol L^{-1} with signal-to-noise (S/N) ratio ≥4. Intra- and Inter-day precisions, expressed as relative standard deviation (RSD) percentages, were ranged from 0.32% to 3.09% and 0.67% to 5.82%, respectively. Finally, it was successfully applied to the determination of amino acids in rat serum with recoveries ranged from 90.8% to 106.0% and RSD percentages ranged from 1.78% to 4.68%, respectively. The results showed that the proposed method provided a shorter elution time, better resolution and sharper peak shapes for all amino acids. Compared with the conventional high performance liquid chromatography (HPLC) methods, even some ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS), the established RRLC method was superior performance.

1. Introduction

In yeast, animals and plants, amino acids play fundamental roles in a multitude of processes. They are not only the basic building blocks of proteins, but also the precursors for biosynthesis of numerous important biological and physiological compounds, which play essential roles in energy metabolism, neurotransmission and lipid transport. Amino acids analyses in many fields, such as in foods, feedstuff, drug quality control, biotechnology and biomedicine are very important [1–4].

Free amino acids can be analyzed by liquid chromatography (LC) [5–7], gas chromatography (GC) [8,9] and capillary electrophoresis (CE) [10–12]. However, the high polarity, low volatility and zwitterionic character of amino acids make simultaneous separation and detection of that so many rather similar compounds more difficult. Usually, derivatization approaches are developed to improve their separation and detection by increasing analytes volatility (for GC–FID/MS analysis) [8,9] or creating amino acid derivatives with strong chromophore/fluorophore groups (for LC or CE–ultraviolet (UV)/fluorescence analysis) [6,10]. Although a number of alternative analytical methods such as CE [11,12], electrochemical [13] and evaporative light scattering detection [14] have been proposed for the determination of underivatized amino acids, these approaches have not been widely used due to limitations including low sensitivity, incompatibility with gradient elution, requiring a volatile mobile phase and inability of analyzing complex biological sample. To some extent, using liquid chromatography combined with tandem mass spectrometry (LC–MS/MS) [15–17] has been shown to be a very sensitive technique for the determination of underivatized amino acids, but apart from involving shorter analysis time and not subjecting to chromatographic interference from the co-eluting compounds, the LC–MS/MS technique is very expensive and not available in many research laboratories. Owing to these reasons, high performance liquid chromatography (HPLC) based on pre- or post-column derivatization is also the most commonly used method for amino acids analysis.

In recent years, with the development of LC technology, LC system has been greatly improved on the basis of traditional HPLC system, i.e., ultra high pressure liquid chromatography (UPLC) and rapid resolution liquid chromatography (RRLC) [18,19]. The latter two systems are greatly superior to the former, and leads to equivalent separations at greatly reduced analysis time and solvent consumption by the combination of shorter columns and smaller particles. RRLC and UPLC methods have shown promising developments in the area of fast chromatographic separations and become the most frequently applied approaches, especially in the field of

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complex biologically derived mixtures analysis, where it is important to increase throughput and reduce analysis costs. Thus, it is advisable to select UPLC or RRLC as chromatographic separation method for the analysis of amino acids.

In the current study, a RRLC method was performed for the simultaneous determination of 23 amino acids in rat serum after pre-column derivatization with 2,4-dinitrofluorobenzene (DNFB). To the authors' knowledge, it was the first time that the RRLC method based on DNFB derivatization used for amino acids analysis. The proposed method provided a shorter elution time, better resolution and sharper peak shapes for all amino acids, and it was successfully applied to the quantitative analysis of 23 amino acids in rat serum with a total elution time of 10 min.

2. Experimental

2.1. Chemicals and reagents

L-Amino acids were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). 2,4-Dinitrofluorobenzene (DNFB) was purchased from Gracia Chemical Technology Co., Ltd. (Chengdu, China). HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). All other chemicals of analytical-reagent grade were purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Ultrapure water used to prepare all aqueous solutions was from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Preparation of standard and derivatization solution

A stock solution containing a mixture of 23 amino acid standards was prepared in water at an individual concentration of 1.0 mmol L^{-1}. Six calibration solutions at different concentrations (1, 10, 25, 100, 250 and 500 μmol L^{-1}) were prepared by serially diluting appropriate amounts of the stock solution with 0.2 M borate buffer (pH 9.0). Similarly, quality control (QC) samples were prepared at four concentrations: 1, 10, 250 and 400 μmol L^{-1} (lower limit of quantification (LLOQ), low, middle and high).

The derivatization solution was prepared by dissolving 0.6052 g DNFB in 50 mL acetonitrile to a concentration of 72 mmol L^{-1}.

2.3. Derivatization

The pre-column derivatization of amino acids with DNFB was a modified version of the reported Refs. [20–22]. To 200 μL of the sample (serum or amino acids standard solutions), 600 μL of acetonitrile was added and vigorously vortexed for 1 min. After the mixture was centrifuged at 13,000 rpm for 10 min at 4 °C, 600 μL of the supernatant was evaporated to dryness at 45 °C under a gentle stream of nitrogen. To the residue, 200 μL of 0.2 M borate buffer (pH 9.0) and 100 μL of DNFB solution were added, and the derivatization reaction was carried out on a water bath (60 °C) for 60 min in the dark. After cooling in ice-cold water, 700 μL of phosphate buffer solution (pH 7.0) was added to the reaction solution. The resulting solution of DNFB derivatives was then briefly vortexed again and transferred through a 0.45 μm nylon filter into auto-sampler vials for injection.

2.4. Rapid resolution liquid chromatography

The RRLC analysis was carried out on an Agilent 1260 series rapid resolution liquid chromatography system (Agilent Technologies, Waldbronn, Germany) which consisted of a G1311B quaternary pump equipped with on-line vacuum degasser, a G1329B refrigerated model SL auto-sampler, a G1316A column oven and a G1315C diode array detector. Chromatographic separations were performed on an Agilent ZORBAX Eclipse Plus C_{18} column (4.6 mm × 50 mm, 1.8 μm; Agilent Technologies). Column temperature was maintained at 45 °C. The mobile phase consisting of 10 mM ammonium acetate solution (A), acetonitrile (B) and methanol (C) was carried out as Table 1 in a gradient elution mode with a flow rate of 1.5 mL min^{-1}. UV detection was set at 360 nm, and the injection volume was 5 μL.

Table 1
Gradient elution program used for the separation of amino acids derivatives under RRLC conditions.

Time (min)	Eluent A (%)	Eluent B (%)	Eluent C (%)
0	90	5	5
4	78	17	5
5	78	22	0
7	67	33	0
9	50	50	0
10	90	5	5

3. Results and discussion

3.1. Derivatization procedure

DNFB was chosen for derivatization since it allows detection in UV region and can react with primary and secondary amines to form yellow colored products through nucleophilic aromatic substitution reaction in aqueous borate buffer and the excess reagent does not interfere. Moreover, DNFB amino acid derivatives have been found to be very stable under common laboratory conditions. Due to these advantageous performances, pre-column DNFB derivatization has been used in our study.

3.2. Optimization of chromatographic separation conditions

Analytical conditions for the analysis of 23 amino acid derivatives using RRLC and traditional HPLC system were optimized and compared, respectively. The best chromatographic separation conditions were presented and discussed here.

The separation of the derivatized amino acids was initially carried out on a Varian 210 high performance liquid chromatograph (Palo Alto, CA, USA) equipped with a 325 UV–vis detector and a Varian Star Chromatographic workstation. Chromatographic separations were carried out on an Agilent Zorbax Eclipse Plus C_{18} (4.6 mm × 250 mm, 5 μm) and a Waters XBridge C_{18} (4.6 mm × 250 mm, 5 μm) column, respectively. The latter showed better performance. A satisfactory resolution of the derivatized amino acids was obtained with a binary mobile phase of 25 mM ammonium acetate solution (A) and acetonitrile (B) (Table 2) at a flow rate of 1.0 mL min^{-1}. A total analysis time of 50 min was obtained at 45 °C after injection of 20 μL.

The subsequent study was focused on optimizing the separation of the derivatized amino acids under RRLC. Chromatographic separations were carried out on an Agilent Zorbax Eclipse XDB C_{18}

Table 2
Gradient elution program used for the separation of amino acids derivatives under HPLC conditions.

Time (min)	Eluent A (%)	Eluent B (%)
0	90	10
25	78	22
35	72	28
40	65	35
45	62	38
46	50	50
50	90	10

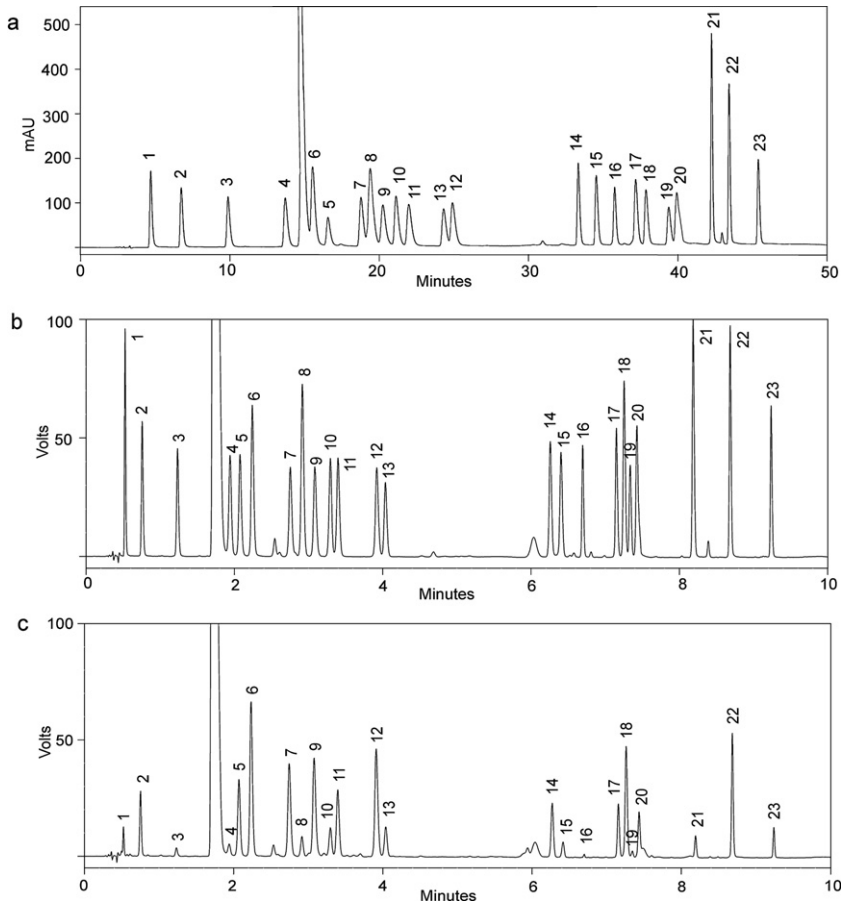

Fig. 1. Chromatograms of (a) HPLC separation of amino acid standards; (b) RRLC separation of amino acid standards and (c) RRLC separation of a rat serum sample. Peak identification: (1) aspartic acid (Asp), (2) glutamic acid (Glu), (3) hydroxyproline (Hyp), (4) asparagine (Asn), (5) glutamine (Gln), (6) serine (Ser), (7) glycine (Gly), (8) arginine (Arg), (9) threonine (Thr), (10) histidine (His), (11) taurine (Tau), (12) alanine (Ala), (13) proline (Pro), (14) valine (Val), (15) cysteine (Cys), (16) methionine (Met), (17) ornithine (Orn), (18) isoleucine (Ile), (19) tryptophan (Trp), (20) leucine (Leu), (21) phenylalanine (Phe), (22) lysine (Lys), and (23) tyrosine (Tyr).

(4.6 mm × 50 mm, 1.8 μm) and an Agilent Zorbax Eclipse Plus C_{18} (4.6 mm × 50 mm, 1.8 μm) column, respectively. The starting point for the optimization of RRLC separation with a mobile phase of 10 mM ammonium acetate: acetonitrile was obtained by a software (Agilent) that estimated RRLC conditions from the best HPLC conditions obtained with the Waters XBridge column. Other parameters that directly affected chromatographic separation such as mobile phase composition, column temperature, injection volume, and flow rate were further studied and optimized under the RRLC conditions. The best resolution of the derivatized amino acids was obtained using chromatographic conditions described in Section 2.4 on the Zorbax Eclipse Plus C_{18} (4.6 mm × 50 mm, 1.8 μm) column. A total analysis time of 10 min was obtained at 45 °C after injection of 5 μL.

3.3. Efficient separation of amino acids by RRLC

Though the best optimized linear gradient elution was applied in the HPLC analysis, it is impossible to reduce the retention times in less than 50 min (Fig. 1a), while RRLC only needed 10 min (Fig. 1b). Analysis throughput under RRLC mode could be four times than conventional HPLC mode. In the RRLC analysis, only a 5 μL of the sample was injected into the column with a flow rate of 1.5 mL min^{-1} to maintain high efficiency and resolution. Although the flow rate corresponding to a higher linear velocity than the conventional HPLC system (1 mL min^{-1}), the higher flow rate with rapid analysis time still significantly reduced the consumption of solvents. In addition, the repeatability of quantitation were assessed and compared between HPLC and RRLC analysis by six consecutive injections of a mixed standard solution (100 μmol L^{-1}). The results indicated the RSD values of retention time and concentrations of each amino acid obtained in the RRLC analysis were both lower than in the HPLC analysis.

The results indicated that RRLC method had several comparative advantages over conventional HPLC method such as analytical speeding, time and solvent saving, high performance and high efficiency. The reduced solvent consumption was also friendly to environment protection. Thus, we eventually selected the RRLC method using Zorbax Eclipse Plus C_{18} (4.6 mm × 50 mm, 1.8 μm) column for the simultaneous determination of 23 amino acids in our study. Typical chromatograms of 23 amino acid standards under the optimal HPLC and RRLC conditions were shown in Fig. 1a and b.

On the other hand, comparing with some recently reported UPLC [23], even UPLC–MS/MS [1,2] methods, our newly developed RRLC method facilitated rapid and satisfactory separations of 23 amino acids within 10 min only. Analysis time was significantly reduced.

3.4. Validation of the RRLC method

After the optimum methods for the quantification of amino acids had been established, the method validation was performed. Calibration curves showed good linearity in the range from 1 to 500 μmol L^{-1} with correlation coefficients of 0.9962 or better for each amino acid. The lower limits of quantification (LLOQ) of all

Table 3
Linearity, precision and recovery results of 23 amino acid derivatives.

Amino acids	Retention time (min)	Calibration curves (1–500 μmol L^{-1})		Precision, RSD (%)						Recovery			
		Equation	Correlation coefficient	Intra-day (n = 6)			Inter-day (n = 18)			Mean (n = 3, %)			RSD (n = 9, %)
				Low	Middle	High	Low	Middle	High	Low	Middle	High	
Asp	0.52	$y = 869.28x + 1326.70$	0.9993	2.42	1.24	0.33	2.89	1.03	1.44	94.9	93.7	90.8	2.23
Glu	0.75	$y = 741.68x + 1303.90$	0.9998	1.43	1.37	0.43	4.92	1.10	1.61	97.5	94.5	93.1	2.40
Hyp	1.23	$y = 708.38x + 461.71$	0.9999	1.68	1.28	0.52	2.82	1.05	1.36	96.1	99.6	100.3	2.32
Asn	1.95	$y = 803.20x + 1083.30$	0.9987	1.54	1.29	0.40	5.82	1.11	2.28	102.0	97.8	98.2	2.33
Gln	2.08	$y = 856.18x + 1469.90$	0.9998	1.18	1.37	0.38	5.15	1.11	1.79	104.1	100.1	98.0	3.07
Ser	2.24	$y = 1259.30x + 1825.60$	0.9999	1.29	1.31	0.39	5.15	1.10	1.59	104.5	100.0	102.3	2.24
Gly	2.76	$y = 838.74x + 2022.30$	0.9980	2.37	1.34	0.40	4.90	1.16	1.86	102.1	94.8	99.6	3.73
Arg	2.92	$y = 1503.60x + 1948.90$	0.9971	1.69	1.30	0.37	4.20	1.03	1.37	97.6	93.5	92.1	3.03
Thr	3.09	$y = 857.72x + 1441.00$	0.9996	2.15	1.16	0.40	5.51	0.98	1.29	103.5	99.1	100.7	2.20
His	3.29	$y = 791.95x + 2097.80$	0.9995	2.83	1.34	0.37	3.09	1.24	1.66	98.2	97.4	93.7	2.52
Tau	3.40	$y = 839.43x + 2407.40$	0.9996	2.25	1.42	0.37	3.77	1.32	1.71	104.3	99.7	97.6	3.45
Ala	3.92	$y = 833.03x + 1553.60$	0.9977	3.09	1.27	0.34	4.00	1.02	1.45	103.1	98.3	100.7	2.37
Pro	4.04	$y = 641.17x + 1619.90$	0.9969	2.98	1.28	0.33	4.54	1.04	1.67	97.9	93.2	91.1	3.71
Val	6.27	$y = 907.92x + 1347.50$	0.9995	1.98	1.32	0.42	5.08	1.10	1.12	100.1	96.4	94.8	2.80
Cys	6.41	$y = 850.62x + 73.09$	0.9992	1.49	1.36	0.40	4.58	1.09	1.84	100.3	94.5	93.5	3.81
Met	6.71	$y = 677.18x - 3600.30$	0.9996	1.13	1.25	0.36	3.28	1.07	1.74	106.0	99.7	101.0	3.23
Orn	7.16	$y = 862.54x + 1301.90$	0.9998	1.52	1.26	0.42	5.23	1.09	0.70	97.4	94.4	92.7	2.51
Ile	7.27	$y = 1190.30x + 2181.30$	0.9985	1.25	1.28	0.42	4.64	1.05	0.76	101.5	100.0	97.9	1.78
Trp	7.35	$y = 570.76x + 240.09$	0.9974	1.17	1.26	0.32	5.56	1.06	1.11	100.3	91.4	95.1	4.68
Leu	7.44	$y = 1272.30x + 2540.90$	0.9962	2.14	1.23	0.44	5.54	1.10	0.67	91.0	94.8	94.0	2.15
Phe	8.20	$y = 1544.50x + 980.76$	0.9986	1.90	1.17	0.41	5.20	0.97	1.46	96.0	93.3	90.8	2.80
Lys	8.69	$y = 1338.70x + 1349.80$	0.9990	1.72	1.53	0.56	5.26	1.30	1.11	103.5	98.4	98.1	3.01
Tyr	9.25	$y = 837.24x - 18.82$	0.9996	1.92	1.33	0.53	3.33	1.36	1.87	103.4	98.4	97.5	3.21

Table 4
Concentrations of amino acids in rat serum.

Amino acids	Concentrations[a] (μmol L^{-1}, n = 10)	Amino acids	Concentrations (μmol L^{-1}, n = 10)
Asp	33.47 ± 1.34	Pro	114.44 ± 12.45
Glu	148.73 ± 7.47	Val	131.95 ± 7.88
Hyp	17.41 ± 4.97	Cys	42.46 ± 2.00
Asn	18.29 ± 1.67	Met	7.83 ± 4.84
Gln	214.85 ± 4.09	Orn	107.88 ± 4.12
Ser	330.92 ± 13.01	Ile	190.38 ± 6.86
Gly	300.81 ± 8.72	Trp	41.81 ± 10.16
Arg	33.75 ± 15.48	Leu	246.42 ± 3.23
Thr	351.42 ± 14.91	Phe	36.66 ± 2.67
His	90.30 ± 5.57	Lys	165.28 ± 19.91
Tau	198.98 ± 20.84	Tyr	56.22 ± 6.62
Ala	359.17 ± 35.33		

[a] The concentrations are presented as mean ± standard deviation.

amino acids were 1 μmol L^{-1} with signal-to-noise ratios (S/N) ≥14. Intra-day (n = 6) and inter-day (3 days) precisions of all amino acids, expressed as relative standard deviation (RSD) percentages, were ranged from 0.32% to 3.09% and 0.67% to 5.82%, respectively. Recoveries of amino acids from rat serum were evaluated by measuring the concentration of each amino acid in rat serum spiked with three different levels (low, middle and high) of QC standard solution. Each sample was tested in triplicate and the average recovery was calculated. Recovery was expressed as [(found concentration − basic concentration)/spiked concentration] × 100%. The recoveries for all the 23 amino acids were ranged from 90.8% to 106.0%, and RSD percentages were ranged from 1.78% to 4.68%, respectively. Linearity, precision and recovery results were summarized in Table 3. Furthermore, the stability of the derivatized amino acids was examined, and the derivatives could be kept at 4 °C for 4 weeks without signals degradation. The results clearly indicated that the DNFB amino acid derivatives were very stable under common laboratory conditions.

3.5. Application

The applicability of the RRLC method for the simultaneous determination of 23 amino acids had been tested by analyzing DNFB amino acid derivatives in rat serum. The chromatogram obtained from a rat serum was shown in Fig. 1c, and the concentration of amino acids in the rat serum was given in Table 4. Amino acids analysis using RRLC allowed good separation and excellent peak shapes with time and solvent consumption decreasing, and it could be considered as a valuable tool for the determination of amino acids in complex biological sample.

4. Conclusion

We have proposed a simple RRLC method for the simultaneous analysis of 23 amino acids in rat serum based on a pre-column derivatizaion with DNFB. The proposed method could be successfully utilized in the analysis of amino acids within a total elution time of 10 min. DNBF had been used as the derivatization reagent due to high UV absorption, very stable derivatives and no interferences from excess reagent or by-products. Compared with the conventional HPLC and some UPLC–MS/MS methods, the newly established RRLC method combining high-throughput chromatography with sensitive DNFB derivatization was superior performance. The efficiency of analysis was significantly improved. In addition, the established method which based on UV detection was economic and available in common laboratories compared with the expensive MS and fluorescence detection.

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