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ORIGINAL ARTICLE

Rapid determination of amino acids in biological samples using a monolithic silica column

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Abstract A high-performance liquid chromatography method in which fluorescence detection is used for the simultaneous determination of 21 amino acids is proposed. Amino acids were derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) and then separated on a monolithic silica column (MonoClad C18-HS, 150 mm × 3 mm i.d.). A mixture of 25 mM citrate buffer containing 25 mM sodium perchlorate (pH 5.5) and acetonitrile was used as the mobile phase. We found that the most significant factor in the separation was temperature, and a linear temperature gradient from 30 to 49°C was used to control the column temperature. The limits of detection and quantification for all amino acids ranged from 3.2 to 57.2 fmol and 10.8 to 191 fmol, respectively. The calibration curves for the NBD-amino acid had good linearity within the range of 40 fmol to 40 pmol when 6-aminocaproic acid was used as an internal standard. Using only conventional instruments, the 21 amino acids could be analyzed within 10 min. This method was found to be suitable for the quantification of the contents of amino acids in mouse plasma and adrenal gland samples.

Keywords Plasma · Adrenal gland · 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) · Temperature gradient

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Introduction

Amino acids are involved in many biological activities that are essential to mammalian life, such as the synthesis of proteins, fatty acids, and ketone bodies. Therefore, there is growing interest in the analysis of amino acids in biological samples (Kaspar et al. 2009; Poinsot et al. 2010). Thus far, many analytical methods in which high-performance liquid chromatography (HPLC)-ultraviolet or fluorescence detection are used to determine amino acids have been reported. However, these methods usually require at least 20–30 min for analysis (Deyl et al. 1986; Aoyama et al. 2004; Langrock et al. 2006; Engel et al. 2006; Gatti et al. 2010). Ion-exchange chromatography with a post-column ninhydrin reaction is commonly used for the automated analysis of amino acids. Recently, faster analytical methods have become preferable. However, conventional particle-packed columns have some limitations, including low separation efficiency resulting from slow mass transfer kinetics and a long analysis time, especially when many types of biomolecules need to be analyzed.

Because of these limitations, considerable research efforts have been directed toward the development of new types of columns. Ultra-performance liquid chromatography (UPLC) and/or mass spectrometry (MS) is one of the solutions. UPLC uses smaller particles (<2 μm) and a higher pressure than does conventional HPLC, leading to faster analysis, better resolution, and higher sensitivity (Wren and Tchelitcheff 2006; Boogers et al. 2008; Armenta et al. 2010). For example, Boogers et al. developed a UPLC method that could be used to quantify 16 amino acids within 10 min (Boogers et al. 2008). With regard to MS detection, amino acids can be determined by MS based on their mass-to-charge ratio (m/z) even when the analytes have the same retention time, implying that

complete separation of amino acids is not necessary and a faster analysis is possible. Although amino acids can be analyzed within 10 min by using UPLC and/or MS (Casetta et al. 2000; Dietzen et al. 2008; Waterval et al. 2009), special and expensive instruments are required.

Monolithic silica column is a promising alternative to conventional particle-packed columns and has attracted increasing attention over the past decade (Guiochon 2007; Unger et al. 2008). Unlike conventional columns, a monolithic silica column consists of a single phase of porous silica. This column has a distinctive bimodal pore structure consisting of macropores (i.d. $\approx$ 2 μm) and mesopores (i.d. $\approx$ 18 nm) that provide sufficient surface area for good separation efficiency. A monolithic silica column can have a total porosity of up to 80%; this is approximately twice that of a conventional column. The above mentioned structural properties enable low flow resistance, fast mass transfer kinetics, high-speed separation, and low column backpressure.

In our previous study, we developed an HPLC method in which a monolithic silica column, MonoClad C18-HS, was used to determine amino acids within 18 min (Song et al. 2011). Because 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was used as the fluorescence derivatization reagent (Watanabe and Imai 1982; Nonaka et al. 2005, 2006; Aoyama et al. 2010), this method was more sensitive than those using other fluorescent derivatization reagents. This method is useful because it requires only an ordinary HPLC system. However, Asp and Cys in plasma samples cannot be determined because the peaks of NBD-Asp and NBD-Cys overlap those of NBD-Cit and NBD-Orn, respectively. Therefore, in this study, 21 common amino acids, namely, Ala, Arg, Asn, Asp, Cit, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Orn, Phe, Pro, Ser, Thr, Tyr, and Val, were separated. We previously used a 250-mm-long monolithic silica column that suffered from high back pressure. In this study, the use of a shorter, 150-mm-long column allowed experiments to be performed at higher flow rates, in turn reducing the analysis time. Using the developed monolithic silica column, the 21 amino acids could be analyzed within only 10 min. This method was successfully applied to mouse plasma and adrenal gland samples.

Experimental

Materials

4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was obtained from Dojindo Laboratories (Kumamoto, Japan). Type-H amino acid standard solution, sodium citrate, citric acid, and sodium perchlorate monohydrate were purchased from Wako Pure Chemical (Osaka, Japan). 6-Aminocaproic acid was obtained from Sigma–Aldrich (St. Louis, MO, USA) and acetonitrile (HPLC grade) was supplied by Honeywell Burdick & Jackson (Muskegon, MI, USA). The water used was purified on a Milli-Q system (Millipore, Bedford, MA, USA).

Fluorescence derivatization procedure and pretreatment of mouse plasma sample

These were performed according to our previous report (Song et al. 2011).

Pretreatment of mouse adrenal gland sample

Male C57/BL6 mice were anesthetized with diethyl ether and the adrenal glands were removed and homogenized with four volumes of 0.4 M perchloric acid containing 0.05% (w/v) EDTA-2Na. The tissue homogenates were centrifuged at 15,000$\times g$ for 5 min at 4°C. The supernatant was stored at −80°C until the assay. Ten microliters of supernatant and 40 μL of internal standard solution (0.1 mM 6-aminocaproic acid) were added to 180 μL of 0.2 M borate buffer (pH 8.5). Then 40 μL of 10 mM NBD-F was added and heated to 60°C for 5 min, and 230 μL of 0.05 M HCl solution was added into the sample in order to stop the reaction.

Chromatographic conditions

The chromatographic system consisted of a pump (PU-1580, Jasco, Tokyo, Japan), a ternary gradient unit (LG-1580-02, Jasco), a degasser (DG-980-50, Jasco), an intelligent column thermostat (CO-1560, Jasco), a fluorescence detector (RF-20A, Shimadzu, Kyoto, Japan), and an integrator (807-IT, Jasco). The fluorescence detection was carried out at an emission wavelength of 530 nm with excitation at 470 nm. The separation was performed using a MonoClad C18-HS (150 mm × 3 mm I.D., GL Sciences, Tokyo, Japan).

Mobile phase A was 25 mM citrate buffer containing 25 mM sodium perchlorate (pH 5.5), and mobile phase B was 25 mM citrate buffer containing 25 mM sodium perchlorate (pH 5.5)/acetonitrile (50/50, v/v). The gradient elution of the mobile phase is shown in Table 1. The temperature of the column oven changed linearly from 30 to 49°C within 10 min. The flow rate was kept at 2.0 mL/min.

Method validation

Standard stock solutions were prepared by dissolving amino acids in 0.1 M HCl solution. Following the

Table 1 Gradient elution system used for analysis of NBD-amino acids

Time (min)	Composition[a] (%)	
	Mobile phase A	Mobile phase B
0	95	5
1.8	76.2	23.8
2.1	76.2	23.8
3.0	68.1	31.9
3.1	50	50
9.5	50	50
9.6	0	100
10	0	100

[a] All the compositions of solvent changed linearly

fluorescence derivatization reactions, NBD-amino acid solutions with different concentrations (0.2, 2, 10, 40, and 200 μM) were injected into the HPLC system to make the calibration. The linear regression of the calibration curves were analyzed by plotting the ratio of the peak heights of the NBD-amino acids to that of internal standard versus the injection amounts of the NBD-amino acids.

In order to determine the accuracy of the method, the standard amino acid solution with different concentrations (0.2, 2, 10, 40, and 200 μM) was added into the mouse plasma sample. Accordingly, the calibration curves could be obtained by the method depicted before. The accuracies were obtained by calculating the ratio of slopes of the standards-spiked plasma sample to the standard amino acids. The precision of the proposed method was investigated by analyzing the same mouse plasma sample for five times in a day and on five consecutive days.

Results and discussion

Optimization of chromatographic conditions

One of the advantages of using a monolithic silica column is that better separation is achieved even at a higher flow rate than that in conventional particle-packed columns. In our preliminary experiments, the theoretical number of NBD-amino acids did not change considerably when the flow rate was increased. To achieve faster analysis under the pressure limits of a monolithic silica column, the following experiments were carried out at a flow rate of 2 mL/min.

We found that the choices of the mobile phase and temperature were significant in the optimization of the chromatographic conditions. First, the mobile phase was optimized. In our previous study, 0.12% trifluoroacetic acid solution was used as the mobile phase, and some amino acids were not separated, as described in the "Introduction". Hence, in this study, various buffer solutions were tested to achieve better separation. Citrate buffer was found to be suitable for separation. However, the peaks of NBD-His, NBD-Gly, and NBD-Gln still overlapped. Sodium perchlorate was used as a mobile phase modifier to optimize the separation of amino acids (Rao et al. 1999; Mant and Hodges 2002). The effect of sodium perchlorate on the separation of NBD-His, NBD-Gly, and NBD-Gln was examined. It was found that the addition of sodium perchlorate led to better separation, and therefore, 25 mM sodium perchlorate was added to the mobile phase.

Next, we examined the effects of temperature on the separation of NBD-amino acids. Figure 1 shows the relationship between the column temperature and the retention times of several NBD-amino acids. As shown in Fig. 1, NBD-Asn and NBD-OH were well separated at 30°C, whereas NBD-Arg and NBD-Ala were well separated at 40°C. Furthermore, increasing the column temperature is effective for reducing the analysis time. Hence, an optimum temperature gradient was used for separation. Accordingly, the optimum chromatographic conditions were as follows: eluent A was 25 mM citrate buffer (pH 5.5) containing 25 mM sodium perchlorate and eluent B was 25 mM citrate buffer (pH 5.5) containing 25 mM sodium perchlorate/acetonitrile (50/50, v/v). The gradient elution of the mobile phase is shown in Table 1. The temperature of the column oven was changed linearly from 30 to 49°C within 10 min.

A representative chromatogram of the 21 NBD-amino acids under the defined optimum conditions is shown in Fig. 2a. The peaks in the chromatogram were identified by the retention times of NBD-amino acids. The separation required only 10 min, which is comparable to other studies in which UPLC and/or MS were used. Because only

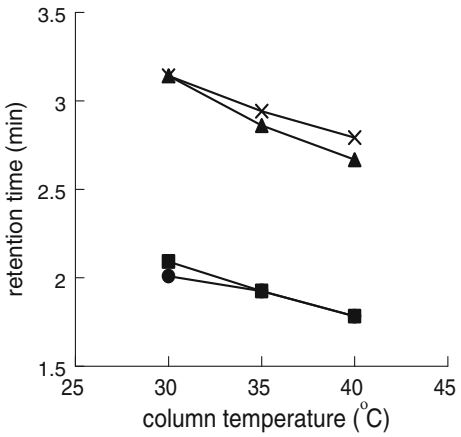

Fig. 1 Relationship between the column temperature and the retention times of NBD-Asn (*filled circle*), NBD-OH (*filled square*), NBD-Arg (*filled triangle*), and NBD-Ala (*cross*)

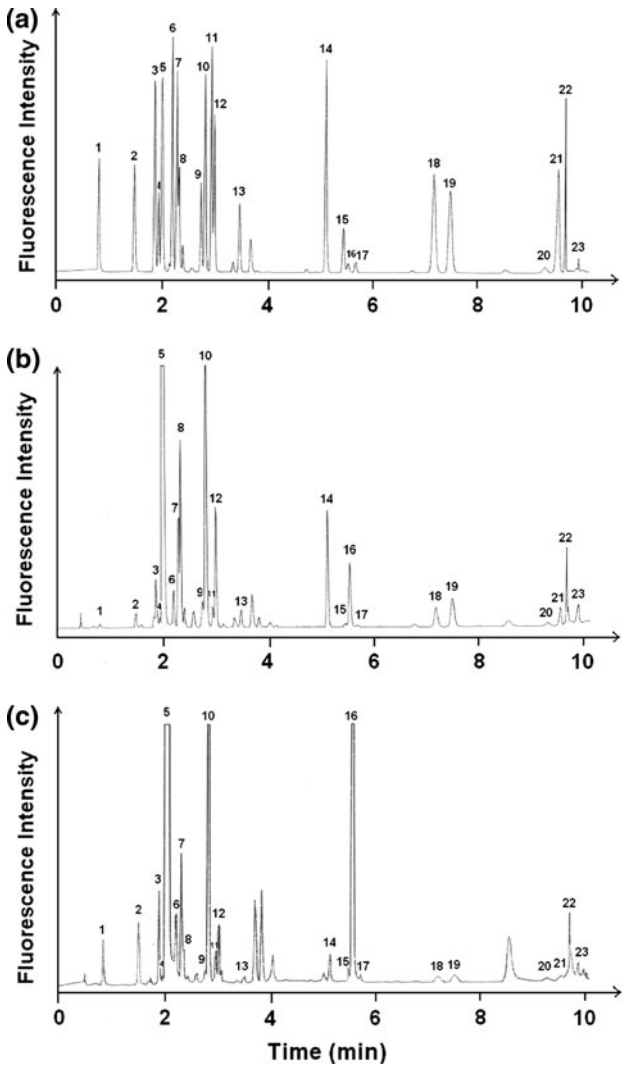

Fig. 2 Chromatogram (**a**) of 21 NBD-amino acid solution, **b** obtained from mouse plasma sample, and **c** obtained from adrenal gland sample. Peaks in the chromatogram: *1* NBD-aspartic acid, *2* NBD-glutamic acid, *3* NBD-serine, *4* NBD-asparagine, *5* NBD-OH, *6* NBD-histidine, *7* NBD-glycine, *8* NBD-glutamine, *9* NBD-citrulline, *10* NBD-threonine, *11* NBD-arginine, *12* NBD-alanine, *13* NBD-proline, *14* NBD-valine, *15* NBD-methionine, *16* NBD-6-aminocaproic acid (internal standard), *17* NBD-cystine, *18* NBD-isoleucine, *19* NBD-leucine, *20* NBD-ornithine, *21* NBD-phenylalanine, *22* NBD-lysine, and *23* NBD-tyrosine

conventional instruments are required in this method, it is very useful for the rapid analysis of amino acids.

Validation of proposed method

The calibration curves for the NBD-amino acids had good linearity in the range of 40 fmol to 40 pmol. The coefficients of correlation were 0.996 or higher for each NBD-amino acid. As shown in Table 2, the limits of detection (LODs) for individual NBD-amino acids ranged from 3.2 to 57.2 fmol at a signal-to-noise ratio of 3, and the limits of quantification (LOQs) ranged from 10.8 to 191 fmol at a signal-to-noise ratio of 10. These values of LOD and LOQ for each NBD-amino acid were comparable to those obtained in our previous studies as well as in other studies (Aoyama et al. 2004; Aoyama et al. 2009; Song et al. 2011).

The precision and accuracy data for the determination of the NBD-amino acids are listed in Table 3. The intra-day precision ($n = 5$) for each NBD-amino acids ranged from 0.37 to 4.7%, and the relative standard deviation for inter-day precision ($n = 5$) ranged from 0.75 to 4.8%. The accuracies for all NBD-amino acids ranged from 91.2 to 110%. These results suggest that the proposed method is suitable for the determination of amino acids in biological samples.

Determination of amino acids in mouse plasma and adrenal gland sample

In mammals, the adrenal glands are endocrine glands that are responsible for releasing hormones including corticosteroids (such as cortisol) and catecholamines (such as epinephrine and norepinephrine) (Hu and Bolten 2006; Fung et al. 2008). Amino acids play many roles in the adrenal glands. Catecholamines are synthesized from Tyr present in the adrenal glands (Tsunoda and Imai 2004; Jirout et al. 2010). Leu and Met are essential in the secretion of adrenocortical hormones (Munro et al. 1965). However, the determination of the amino acids present in adrenal glands has seldom been reported. Hence, the proposed method was applied to determine the amino acids in an adrenal gland sample as well as a mouse plasma sample. The chromatograms shown in Fig. 2b and c were obtained from mouse plasma and an adrenal gland sample, respectively. The concentrations of the amino acids are shown in Table 4. The values obtained in the present study are in agreement with those of previous studies (Aoyama et al. 2004; Gu et al. 2008; Jambor and Molnar-Perl 2009; Kelly et al. 2010; Song et al. 2011).

Conclusion

An HPLC method in which a monolithic silica column (MonoClad C18-HS, 150 mm × 3 mm i.d.) was used to determine amino acids in mouse plasma and adrenal gland samples was developed. The amino acids were pre-column derivatized with NBD-F. During the analysis, a specific temperature gradient was used to optimize the separation conditions. After optimization, 21 NBD-amino acids could be analyzed within only 10 min; this process was much faster than those used in previous methods. The developed

Table 2 Limit of detection (S/N = 3) and limit of quantification (S/N = 10) of individual NBD-amino acids

Amino acid	Limit of detection (fmol)	Limit of quantification (fmol)	Amino acid	Limit of detection (fmol)	Limit of quantification (fmol)
Asp	11.7	39.0	Pro	3.2	10.8
Glu	14.8	49.3	Val	9.5	31.8
Ser	3.7	12.2	Met	50.1	167
Asn	9.4	31.2	Cys	20.3	67.8
His	13.4	44.5	Ile	18.8	62.7
Gly	3.5	11.5	Leu	21.1	70.3
Gln	3.9	13.2	Orn	57.2	191
Cit	8.4	27.9	Phe	25.0	83.4
Thr	7.3	24.2	Lys	10.5	35.1
Arg	8.9	29.9	Tyr	19.0	63.6
Ala	6.4	21.5			

Table 3 Precisions of the proposed method for the determination of mouse plasma sample

Amino acid	Intra-day precision (%) ($n = 5$)	Inter-day precision (%) ($n = 5$)	Accuracy (%)	Amino acid	Intra-day precision (%) ($n = 5$)	Inter-day precision (%) ($n = 5$)	Accuracy (%)
Asp	2.17	4.31	100.2	Pro	1.43	0.75	105.8
Glu	1.92	3.32	102.3	Val	0.79	2.25	94.0
Ser	0.86	2.38	102.2	Met	2.29	2.37	106.0
Asn	2.77	3.79	91.8	Cys	1.32	2.28	94.2
His	2.77	4.64	95.8	Ile	0.37	2.49	105.2
Gly	2.42	3.94	94.8	Leu	0.53	1.31	95.2
Gln	2.46	4.51	91.2	Orn	3.55	1.94	91.5
Cit	1.84	4.17	94.7	Phe	0.92	1.27	92.0
Thr	1.51	4.81	94.3	Lys	4.60	3.06	95.4
Arg	1.61	2.20	109.8	Tyr	4.66	2.65	95.3
Ala	0.83	4.34	93.0				

Table 4 Concentrations of amino acids in mouse plasma sample and adrenal gland sample

Amino acid	Concentrations in mouse plasma sample (μM) ($n = 3$)	Concentrations in adrenal gland sample (pmol/mg tissue) ($n = 3$)	Amino acid	Concentrations in mouse plasma sample (μM) ($n = 3$)	Concentrations in adrenal gland sample (pmol/mg tissue) ($n = 3$)
Asp	2.93 ± 0.36	228 ± 18.2	Pro	72.0 ± 8.3	134 ± 6.7
Glu	33.6 ± 4.0	286 ± 37.1	Val	156 ± 21.8	114 ± 8.0
Ser	57.0 ± 4.4	173 ± 36.0	Met	17.1 ± 2.7	230 ± 20.7
Asn	13.6 ± 1.3	80.6 ± 6.5	Cys	46.3 ± 6.3	114 ± 9.0
His	52.5 ± 4.8	333 ± 20.0	Ile	69.3 ± 8.3	32.7 ± 2.3
Gly	163 ± 25.9	214 ± 29.3	Leu	102 ± 8.9	39.2 ± 5.1
Gln	390 ± 48.7	70.4 ± 7.1	Orn	65.6 ± 6.0	298 ± 23.9
Cit	37.6 ± 4.8	131 ± 11.8	Phe	46.6 ± 5.0	35.4 ± 4.3
Thr	142 ± 25.1	1190 ± 140	Lys	176 ± 13.4	223 ± 34.5
Arg	28.6 ± 2.1	77.8 ± 8.6	Tyr	103 ± 9.2	63.1 ± 9.7
Ala	182 ± 20.7	88.1 ± 9.3			

method requires no special and expensive instruments, and amino acids can be rapidly analyzed using a monolithic silica column instead of a particle-packed column. The method can potentially find wide applications in routine analysis and contribute to research on the physiological actions of amino acids.

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Conflict of interest The authors declare that they have no conflict of interest.

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