論文の内容の要旨

Development of liquid chromatography method for fast and quantitative analysis of amino acids and related compounds
(液体クロマトグラフィーを用いたアミノ酸類化合物の高速定量法の開発)

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Introduction

The identification of essential compounds in biological samples is a hot topic in the field of life science, because the analysis of the compounds in biological samples could provide a new insight into physiological, developmental, and pathological status of a biological system. High-performance liquid chromatography (HPLC) is the most common tool for analyzing biological samples because it requires little equipment and is highly reliable; however, current separation efficiency could not meet the requirements for the fast and quantitative determination of biological samples. Therefore, development of a fast and quantitative liquid chromatography method for the compounds in the biological samples is urgently necessary. In my research, amino acids and related compounds, which are involved in many biological activities, were chosen and analyzed.

In the first part of my thesis, two new types of column, including monolithic silica column and core-shell column, were applied for the analysis the 21 amino acids.

Secondly, pillar array column, which has not been applied for the quantitative analysis, would be applied for fast and analysis of branched-chain amino acids (BCAAs, valine, isoleucine, leucine) in biological samples.

Thirdly, in order to apply the pillar array column for analysis of complex compounds in biological sample, a gradient elution system was fabricated before the pillar array column on the microchip. The developed gradient elution system was applied for the separation of NBD-aliphatic amines.

Experimental and Results

1. Analysis of amino acids with monolithic silica column and core-shell column

To date, many HPLC methods have been developed for the analysis of amino acids in biological samples, however, a long analysis time was needed. In order to achieve a faster analysis, several new columns have been developed. Monolithic silica column is a single piece made of porous cross-linked silica. Macropores with a diameter of 1~10 μm have a low flow resistance, which ensure high-speed separations at a relatively low column backpressure. Mesopores with a pore diameter of about 10~20 nm could provide an efficient separation because of sufficient surface area. The core-shell particle column is packed with particles in which solid cores with surrounding porous silica shells. This semi-porous silica column provides a shorter diffusion path for a solute to minimize peak broadening and allows high speed separations with lower operating pressure of the column.

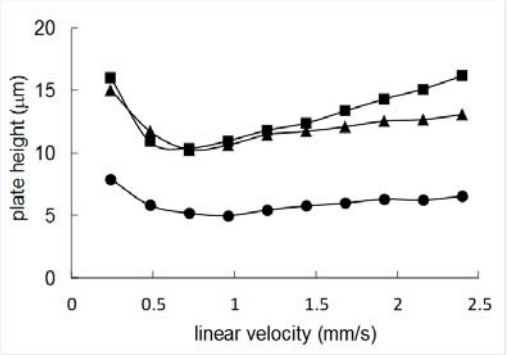

Figure 1. van Deemter plotting for NBD-Ser using a conventional particle-packed column (ODS-4 column) (■), a monolithic silica column (MonoClad C18-HS) (▲), and a core-shell particle column (Kinetex C18-100A) (●)

Before analysis, the amino acids was fluorescently labeled by 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) because of its high reactivity and sensitivity. The van Deemter curves of NBD-Ser by the three columns (conventional particle-packed column, monolithic silica column, and core-shell particle column) were plotted as to compare the separation efficiencies. The experiments were performed with linear velocities in the range of 0.24 and 2.4 mm/s. As shown in Figure 1, the minimal theoretical plate height of NBD-Ser using the core-shell particle column was 5.0 μm, which was only half of the values obtained by the conventional particle-packed column (10.4μm) and monolithic silica column (10.2μm). For the core-shell particle and monolithic silica columns, theoretical plate height did not vary with an increase in the linear velocity. The core-shell particle column has higher separation efficiencies comparing with conventional particle-packed column and monolithic silica column.

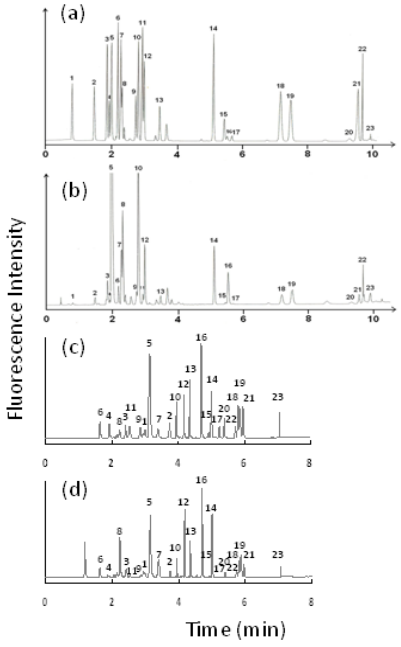

Figure. Chromatogram of (a) 21 NBD-amino acid solution and (b) mouse plasma samples by monolithic silica column (c) 21 NBD-amino acid solution and (d) mouse plasma samples by core-shell column. Peaks in the chromatogram: 1, NBD-Asp; 2, NBD-Glu; 3, NBD-Ser; 4, NBD-Asn; 5, NBD-OH; 6, NBD-His; 7, NBD-Gly; 8, NBD-Gln; 9, NBD-Cit; 10, NBD-Thr; 11, NBD-Arg; 12, NBD-Ala; 13, NBD-Pro; 14, NBD-Val; 15, NBD-Met; 16, NBD-6-aminocaproic acid (internal standard); 17, NBD-cystine; 18, NBD-Ile; 19, NBD-Leu; 20, NBD -Orn; 21, NBD-Phe; 22, NBD-Lys; and 23, NBD-Tyr.

The analyses of 21 NBD-amino acids in mouse plasma sample were performed on a monolithic silica column and core-shell column within 10 and 7 min, respectively (Figure 2). The analysis time was greatly shortened comparing with previous studies. The concentrations determined by the two methods are similar with the previously reported data. The methods were proved to be fast, accurate, and were applicable to the analysis of amino acids in biological samples. These methods can be potential tools to clarify the physiological roles of amino acids in biological activity and the diagnosis of disease in clinical field.

2. Rapid and quantitative analysis of BCAAs in biological samples using a pillar array column

With the development of precise fabrication technologies, pillar array columns with almost perfectly ordered structures have been fabricated. These columns have effectively eliminated eddy diffusion resulting from non-ideal particle packing, which was the main factor contributing to band broadening. However, short pillar array column on the microchip could not provide a high theoretical plate number which could ensure good resolution for the analysis of biological samples. In the previous study, a longer pillar array column was folded with the low-dispersion turn, which could efficiently reduce the turn-induced dispersion, and a better separation of several biological compounds with similar polarity property was achieved. To date, pillar array column have not been used for quantitative analysis.

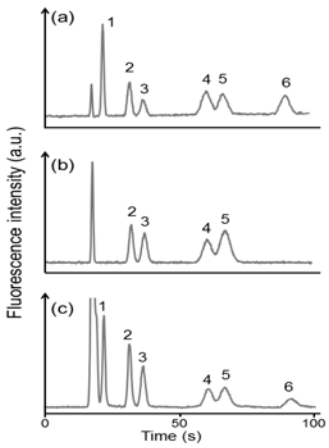

Figure 3. Chromatograms of (a) NBD-BCAAs solution, (b) sports drink, and (c) human plasma sample. Peaks: 1, NBD-Pro; 2, NBD-Val; 3, NBD-6-aminocaproic acid; 4, NBD-Ile; 5, NBD-Leu; and 6, NBD-Phe

In order to verify pillar array column could be applied for quantitatively analysis of compounds in biological samples, the BCAAs valine, leucine, and isoleucine, which are important disease markers for chronic liver disease, diabetes, and heart disease, were selected and their concentrations in human plasma sample were quantitatively determined.

The pillar array column had a length of 110 mm, and was folded with low-dispersion turns to fit on a 20 × 20 mm microchip. The pillars in the column were squares with sides of 3 μm with an inter-pillar distance of 2 μm. The optimum chromatographic conditions to achieve rapid analysis of NBD-BCAAs were investigated. The optimized conditions were as follows: the mobile phase was water–acetonitrile–TFA (92:8:0.02, v/v/v), flow rate was 2 μL/min, and the detection point was set near the outlet of the separation channel. Under the optimized conditions, the analysis could be completed in 100 s. Figure 3(a) shows a chromatogram of the NBD-BCAAs.

To eliminate variation resulting from the uncontrolled injection volume, 6-aminocaproic acid was added as an internal standard, which improved the reproducibility of NBD-Val from 28.4% to 3.23%. The calibration curves for all NBD-BCAAs were linear in the range of 0.4 to 20 μM. The developed method was then used for the determination of BCAAs in sports drink and human plasma samples; the corresponding chromatograms are presented in Figure 3(b) and (c), respectively. Both the intra- and interday precisions (n = 5) were below 5%. The accuracies for the three BCAAs ranged from 90.2% to 99.6%. The concentrations of BCAAs determined by the pillar array column were similar to those obtained using a conventional column. Therefore, the pillar array column can be used to analyze the components in biological samples, and could become an essential tool for clinical diagnosis.

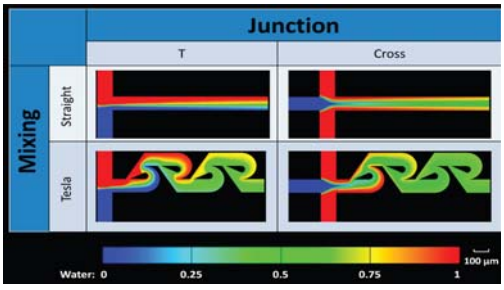

Figure 4. Simulation results determined by CFD analysis of the mixing of water and ethanol in the T-straight mixer, the cross-straight mixer, the T-Tesla mixer, and the cross-Tesla mixer.

3. Development of a gradient elution system for pillar array column

Because the compounds in real biological samples are quite complex and exhibiting big differences in polarity, a gradient elution system that can accelerate the elution of strongly retained compounds should be integrated with pillar array column for fast analysis. To eliminate the delay that occurs in off-chip gradient elution systems, a micromixer with high mixing efficiency was positioned immediately before the separation channel. First, the mixing efficiencies of four mixers, which were combinations of different inlet junction and mixing structures, namely, T-straight, T-Tesla, cross-straight, and cross-Tesla, were investigated by both computational fluid

dynamics simulation (Figure 4) and experiments involving mixing two solutions. The results showed that the cross-Tesla mixer achieved the best mixing efficiency of the mixers investigated, and two solutions were mixed efficiently after running for 3 mm in the mixer. Therefore, a cross-Tesla mixer (18 mm), injection channel, and separation channel with a pillar array (58 mm) were fabricated on a 20 × 20 mm microchip. An overview of the microchip is presented in Figure 5.

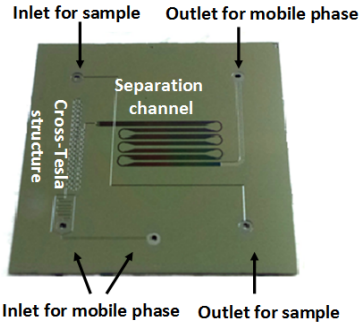

Figure 5. A photograph of the fabricated microchip.

To confirm that the gradient elution system operated as intended, solutions of fluorescein in methanol and water were pumped into the microchip with gradient elution. The fluorescence intensity remained stable during the 30 s of isocratic elution. When the gradient elution started, the fluorescence intensity increased linearly with a delay time of only 7 s. The results showed that the two solutions were mixed effectively before entering the separation channel.

Two coumarin dyes, Coumarin 525 and 545, were separated using the gradient elution system. These two dyes were separated in about 55 s under isocratic conditions. The analysis was faster when the gradient time was shortened. When the gradient time was 30 s, the analysis was achieved in just 30 s. The peak width of Coumarin 545 was 6.9 s under isocratic conditions. However, when the gradient time was 30 s, the peak width of Coumarin 545 decreased to just 2.1 sec, demonstrating that gradient elution considerably improved the separation efficiency of Coumarin 525 and 545.

The gradient elution system was then used to separate four kinds of NBD-aliphatic amines because they are involved in many biological reactions, such as the biodegradation of proteins, amino acids, and other biological compounds. As shown in Figure 6, when the separation was performed under isocratic elution, NBD-octylamine was not eluted after 1300 s. When gradient elution was used, the four NBD-aliphatic amines could be analyzed in 110 s. The peaks also became sharper compared with those under isocratic elution conditions. These results show that the developed chip could be a useful tool to analyze compounds with different polarity in biological samples. The quantitative analysis of biological samples under gradient elution is in progress.

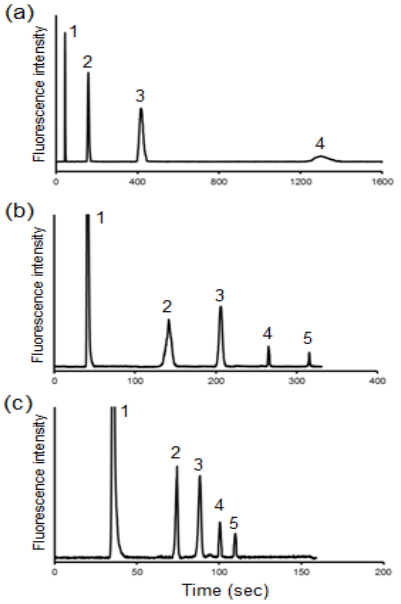

Figure 6. Chromatograms of separation of NBD-amines obtained under isocratic elution (a), mobile phase: water/acetonitrile/TFA (90/10/0.1) and gradient elution, mobile phase A: water/acetonitrile/TFA (90/10/0.1), mobile phase B: water/acetonitrile/TFA (10/90/0.1), gradient program (0 s, 100/0; end, 0/100); gradient time: (b), 450 s; and (c), 90 s. Peaks: 1, NBD-OH; 2, NBD-pentylamine; 3, NBD-hexylamine; 4, NBD-heptylamine; 5, NBD-octylamine.

Conclusion

In this research, analyses of 21 amino acids in biological samples were achieved in 10 and 7 min by using monolithic silica column and core-shell column. With the help of internal standard, the pillar array column realized the fast and quantitative analysis of BCAAs in sports drink and human plasma sample. A gradient elution system was integrated with a pillar array column for fast analysis of complex compounds in biological samples. Compared with the isocratic elution system, more compounds could be eluted and analysis time was also greatly shortened. The developed method is promised to be a potential tool for the fast and quantitative analysis of complex biological samples.