Abstract

In this study, the application of a new type of monolithic silica column to the analysis of amino acids in human plasma samples has been investigated. The monolithic silica column (3.0 × 150 mm, MonoTower C18) provided good separation of the amino acids derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). Although the separation ability of this column was not much improved in comparison with the previous monolithic silica columns, it was similar with that of a 3 μm particle packed column. The column with length 150 mm had the same separation property as a tandem connection of columns of lengths 50 mm and 100 mm. After 1000 injections of human plasma samples, the peak widths for the NBD-amino acids were increased by only 8%. Thus, the newly developed monolithic silica column can be potentially applied for faster analysis and/or more efficient separation of biological compounds.

Keywords: Fluorescence; 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F); Plasma; Durability

1. Introduction

There is an increasing demand for improving the performance of bioanalytical methods with faster analysis and/or more efficient separation techniques. Consequently, there has been significant progress in column technology with the use of smaller particles, core-shell silica particles, monolithic columns, and chip-based liquid chromatographic separations [1-6].

Monolithic silica columns are composed of a continuous piece of porous cross-linked silica and these have several advantages over conventional particle-packed columns [7]. Monolithic silica columns have two types of pores, namely, macropores and mesopores. The size and structure of these pores can be changed, which influences the separation performance [8]. The macropores lower the flow resistance, which enables high-speed separations under low back pressure in comparison with particle-packed columns, leading to a great reduction in the analysis time [7-9]. Furthermore, a thin framework and a convective flow result in the rapid diffusion of analytes to the surface area, achieving high separation efficiency [9].

Commercial monolithic silica columns have been available since 2000. Very recently, the next generation of monolithic silica columns, by the name of MonoTower (Fig. 1), has been developed and is now commercially available. It has smaller macropore (0.95 μm) and mesopore (11 nm) sizes and an increased phase ratio (total porosity < 0.7) compared to the previous commercially available monolithic silica columns. With these unique properties, the MonoTower is expected to result in a higher separation efficiency and a larger retention factor. As there are always concerns about the monolithic columns such as column durability and column-to-column reproducibility, it is crucial to investigate such properties of the new generation monolithic silica columns, in addition to their separation efficiencies. Furthermore, as far as we know, there are no reports to date that discuss the application of this column to the analysis of biological compounds and/or biological fluids such as blood plasma and urine samples. Therefore, in this study, we applied the MonoTower column to the chromatographic analysis of amino acids in human plasma samples.

2. Experimental

2.1. Chemicals

4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was received: 28 August 2017
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obtained from Dojindo Laboratories (Kumamoto, Japan) and glutamine and 6-aminocaproic acid were purchased from Kyowa Hakko Kogyo Co. (Tokyo, Japan). Trifluoroacetic acid (TFA) and type-H amino acids standard solution were acquired from Wako Pure Chemical Industries (Osaka, Japan) while acetonitrile (HPLC grade) was obtained from Merck KGaA (Darmstadt, Germany). A Milli-Q system (Merck Millipore, Darmstadt, Germany) was used to purify water. Asparagine and human plasma were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical reagent grade.

2.2. Sample pretreatment
To 40 μL of the human plasma or amino acid solution, 40 μL of an internal standard solution (20 μM 6-aminocaproic acid), 160 μL of methanol, and 160 μL of acetonitrile were added. The mixture was centrifuged at 10,000×g for 5 min at 4°C. Subsequently, 20 μL of the supernatant was evaporated to dryness under reduced pressure.

2.3. Fluorescence derivatization
Sample derivatization was performed according to previous studies [10-12]. A volume of 20 μL of the sample was added to 180 μL of a 0.2 M borate buffer (pH 8.5). Subsequently, 40 μL of a 10 mM NBD-F solution was added to the mixture and heated to 60°C for 5 min. After cooling the reaction mixture in ice water, 260 μL of a 50 mM HCl aqueous solution was added to the reaction mixture. This was followed by the injection of 10 μL of the resultant solution into the HPLC system.

2.4. Apparatus
An HPLC system consisting of an HPLC pump (PU-980, Jasco, Tokyo, Japan), a ternary gradient unit (LG-1580-02, Jasco), a degasser (DG-980-50, Jasco), an autosampler (AS-1550, Jasco), a column oven (CO-1560, Jasco), and a fluorescence detector (RF-20A, Shimadzu, Kyoto, Japan) was used in this study. Fluorescence detection was performed at 530 nm with excitation at 470 nm. Chromato-Pro (Run Time Corporation, Hachioji, Japan) was used to analyze the chromatograms.

2.5. Chromatographic separation conditions
The chromatographic separation was performed by using a MonoTower C18 (3.0 × 150 mm or 50 + 100 mm, GL Sciences, Tokyo, Japan) column. The mobile phase (A) was a mixture of water/acetonitrile/TFA (90/10/0.08, v/v/v), and the mobile phase (B) consisted of acetonitrile/TFA (100/0.14, v/v). The gradient conditions for the separation of NBD-amino acids were as follows: 0–10 min at 0% mobile phase B, 10–15 min linear gradient from 0% to 30%, 15–25 min at 30%, 25–26 min linear gradient from 30% to 100%, 26–30 min at 100%. The flow rate was 0.6 mL/min.

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Fig. 1. Photograph of the MonoTower columns

Fig. 2. Chromatograms of the standard solutions ((a) and (c)) and that of the human plasma sample (b). While chromatograms (a) and (b) were obtained using a 150 mm column, chromatogram (c) was obtained by using 50 + 100 mm columns. Peaks in the chromatogram: 1 NBD-histidine, 2 NBD-asparagine, 3 NBD-glutamine, 4 NBD-serine, 5 NBD-aspartic acid, 6 NBD-glycine, 7 NBD-glutamic acid, 8 NBD-threonine, 9 NBD-alanine, 10 NBD-proline, 11 NBD-methionine, 12 NBD-valine, 13 NBD-cystine, 14 NBD-lysine, 15 NBD-isoleucine, 16 NBD-leucine, 17 NBD-phenylalanine, 18 NBD-tyrosine, and 19 NBD-6-aminocaproic acid (internal standard).
and the column oven was set at 40°C.

2.6. Validation study

Amino acids were dissolved in 0.1% TFA aqueous solution for stock solutions. The calibration standards for amino acids with different concentrations (0, 1.5, 7.5, 15, 30, and 60 μM) were prepared from the stock solutions. Linear calibration curves were produced by plotting the ratio of the peak area of the NBD-amino acids to that of the internal standard. The limits of detection (LODs) and limits of quantification (LOQs) for the respective NBD-amino acids were calculated as the injection amounts at signal-to-noise ratios of 3 and 10, respectively.

3. Results and discussion

3.1. Separation of NBD-amino acids

Based on our previous study using a monolithic silica column (MonoClad C18-HS) [10], the separation of NBD-amino acids was first performed by using the mobile phases of the solvent mixture A, consisting of water/acetonitrile/TFA (90/10/0.12, v/v/v) and the solvent mixture B, comprised of water/acetonitrile/TFA (10/90/0.12, v/v/v) for gradient elution. Although the retention ability of MonoTower column was similar to that of the MonoClad column, the NBD-amino acids could not be separated well. Hence, the separation conditions were optimized basically by changing the TFA concentration, which was effective on the separation of NBD-amino acids [13]. Finally, the optimized mobile phase and gradient conditions were as described in Experimental section. Thus, the NBD-amino acids were separated by using the optimized separation conditions, as shown in Fig. 2(a).

3.2. Chromatographic property of the MonoTower column

To the best of our knowledge, this is the first report wherein a MonoTower column has been applied for the analysis of biological compounds. We also compared the separation property of the column with those of a monolithic silica column (MonoClad) and a conventional particle packed column (Inertsil ODS-4, 3.0 × 150 mm, 3 μm). It is worth noting that the previous data was obtained on a different liquid chromatography system and different mobile phase conditions were used, which causes difficulty for a complete comparison.

The plate heights were compared among the three columns for the peak of NBD-Gly. The value was 12 μm for all columns, which indicated that the three different columns have similar separation ability. Since the MonoTower column has a smaller macropore and mesopore sizes compared to the previously reported columns, it was expected that the separation efficiency of the MonoTower column would be better than that of the MonoClad column. However, the result was not as expected, probably because the separation efficiency depends on the sample compounds and its difference might be too small to be detected. Although the separation ability of the MonoTower column was not significantly better than that of the MonoClad column, it was equivalent to that of the 3 μm particle packed column.

One of the advantages of the monolithic columns is the lower pressure drops than the particle packed columns. In fact, the pressure drop with the flow rate of 0.6 mL/min and the initial mobile phase was around 90 bar. As this property is dependent on the mobile phase conditions, the pressure-drop per column length of MonoTower was similar with those of the previously reported monolithic columns, and was approximately 70% of the 3 μm particle packed columns. Although the increase in the flow rate was not investigated in this study, the low-pressure drop property of the column can be used at higher flow rates or longer column lengths, which should result in a higher plate number.

3.3. Validation data

A linearity correlation was found between the peak area ratios and the concentrations of the standards at the injection amounts of 60–2400 fmol with the correlation coefficient of >0.998. The LODs and LOQs of NBD-amino acids were 0.6–17 fmol/injection and 1.9–57 fmol/injection, respectively. These values are similar with those reported in previous studies with monolithic silica columns [10], particle packed columns [14], and core-shell particle columns [15].

3.4. Application to human plasma samples

As mentioned in the Introduction, the MonoTower column has not been applied to biological samples. Therefore, amino acids in human plasma samples were analyzed by using the column. Figure 2(b) shows the chromatogram of the human plasma sample under the optimized conditions. Amino acid concentrations in the human plasma samples were in the range 19.2–331 μM, which is quite similar with that described in the previous reports [16,17].

3.5. Investigation of tandem columns

As this cartridge type column has several different lengths, the 150 mm long column was replaced with two tandem columns of lengths 50 mm and 100 mm. Subsequently, it was confirmed that the 150 mm long column had the same separation ability as the tandem columns. As shown in Fig. 2(c), the tandem columns showed identical retention of the NBD-amino acids as observed for the 150 mm column. This property can be exploited if the column needs to be replaced when it is damaged. The durability of the 50 + 100 mm tandem
The column was examined by repeated injections of human plasma samples. The plasma samples were injected 1000 times into the HPLC system, and chromatograms of the standard were taken at random points during the experiments. The peak widths were calculated for each NBD-amino acid. Figure 3 shows the changes in the peak widths of NBD-Ala and NBD-Val. There was no increase of peak widths after 400 injections of plasma samples, which was only 8% after 1,050 injections. Similar trends were observed for the other NBD-amino acids. These results suggest that the column can be used for at least 1000 injections of biological samples. Although it was not performed in this study, only the 50 mm long column can be changed when the column is damaged, which is more cost effective than changing of the column of 150-mm length.

4. Conclusion

Amino acids labelled with a fluorescent group were separated on a newly developed monolithic silica column (MonoTower). Although the separation ability of this column was not much better than that of the previously reported monolithic silica columns, its performance was similar to that of a 3 μm particle-packed column, albeit with a lower pressure-drop. As the column is cartridge type, 50 + 100 mm tandem columns could be used instead of a single column of length 150 mm with the same separation properties. It was possible to use this column for 1000 injections of the plasma samples. Thus, it was shown that the MonoTower column could be applied for the analysis of various compounds in biological samples.

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