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Determination of glutamine and serine in rat cerebrospinal fluid using capillary electrochromatography with a modified photopolymerized sol–gel monolithic column

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Abstract

Capillary electrochromatographic separations of amino acid mixtures were studied using two modified porous photopolymerized sol–gel monolithic columns. One was modified with dimethyloctadecylchlorosilane (DMOS), and the other was modified with DMOS, followed by chlorotrimethylsilane to end-cap residual silanol groups. Prior to separation, amino acids were derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole using as a mobile phase 50 m*M* phosphate (pH 2.5), water, and acetonitrile in the ratio of 1:1:8. Five derivatized amino acids (Asn, Phe, Ala, Ile, and Leu) were separated within 7 min. Theoretical plate numbers varied between 58 700 and 105 000/m. This separation method with the end-capped monolithic column was applied to rat cerebrospinal fluid. The dominant amino acid found was Gln at a concentration of 420 μ*M* along with small quantities of Ser (54 μ*M*).

Keywords: Monolithic columns; Sol–gel; Glutamine; Serine; Amino acids

1. Introduction

Capillary electrochromatography (CEC), which is a hybrid separation technique of HPLC and CE [1,2], enables the analysis of small volumes of sample with short run times because of the high separation efficiencies that can be achieved. A variety of analytes have been separated by CEC. They include biologically important molecules such as proteins, peptides, and amino acids. CEC is suitable for the analysis of real samples whose limitations include small sample volumes, high sample complexity, and low analyte concentrations. But to date, only a few analyses of real samples by CEC have been reported [3–5].

Because capillaries have a very narrow diameter (typically less than 100 μm), elaborate work including the fabrication of on-column frits is required to prepare a stationary phase with good reproducibility and homogeneity. Bubble formation or deterioration of the separation efficiency has been observed in frit sections [6–8]. To overcome these difficulties and

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problems of packed CEC column preparation, monolithic CEC columns were first introduced by Hjertén and co-workers [9] and developed further by Svec and Fréchet [10]. Monolithic CEC columns are easily prepared and show good chromatographic performances [11]. Many monolithic columns for CEC have been fabricated using a variety of monomers, such as acrylamide [12–18], methacrylate [19–22], and alkoxysilane [23–29].

Recently at Stanford University, we prepared monolithic columns from methacryloxypropyltrimethoxysilane (MPTMS) [30,31]. MPTMS, which contains both methacrylate and alkoxysilane groups, was used to create a photopolymerized sol–gel (PSG) monolith in a single-step reaction. The PSG monolith showed good separation of some neutral compounds, such as alkylbenzenes and polycyclic aromatic hydrocarbons. The separation of charged compounds, however, was not satisfactory when using this PSG monolithic column because charged compounds were adsorbed onto the residual silanol groups of the PSG monolith surface. Consequently, we modified the PSG monolith with silane-coupling reagents. The modified PSG was effective for the separation of both charged and uncharged compounds [32].

In this paper, dimethyloctadecylchlorosilane (DMOS), which is a typical coupling reagent to prepare ODS packing materials for HPLC, was chosen for use as a silane-coupling reagent. An end-capping reaction with chlorotrimethylsilane (CTMS) was also tried after the modification of PSG monolith with DMOS. The end-capping reaction is often carried out for many ODS packings that are used in HPLC columns to prevent adsorption of analytes by the residual silanol groups. The separation properties of an octadecyl PSG monolithic column (non-end-capped) and an end-capped PSG monolithic column were compared by CEC.

Amino acids were chosen as charged samples, because these are very important components in many biological and food samples. Rat cerebrospinal fluid (CSF) is an example of a real biological sample. Techniques such as HPLC, CE, and CE/microdialysis have been used for the analysis of amino acids in rat CSF [33–35]. In fact, the measurement of the Gln concentration in CSF has been used to diagnose encephalopathies of hepatic origin [35]. All amino acids are derivatized with a fluorogenic reagent, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), to obtain highly sensitive detection. The separation properties of derivatized amino acids on these monolithic columns were examined and the modified PSG monolithic column was applied to the determination of amino acids in rat CSF.

2. Experimental

2.1. Materials and chemicals

UV-transparent coated fused-silica capillaries (75 μm I.D.×375 μm O.D.) were purchased from Polymicro Technologies (Phoenix, AZ, USA). MPTMS, DMOS, NBD-F, and CTMS were purchased from Tokyo Kasei (Tokyo, Japan). Toluene, acetonitrile, thiourea, ammonium acetate, and trifluoroacetic acid were from Kanto Kagaku (Tokyo, Japan). Amino acids were purchased from Sigma–Aldrich (Milwaukee, WI, USA). Diethylamine, phosphoric acid, sodium dihydrogenphosphate dehydrate, and boric acid were from Wako (Osaka, Japan). Irgacure 1800 was donated from Ciba (Tokyo, Japan). The water was purified by a Milli-Q system (Nippon Millipore, Tokyo, Japan).

2.2. Apparatus

All the CEC experiments were carried out at the University of Shizuoka using a capillary electrophoresis system (Beckman model P/ACE System 5510, Fullerton, CA, USA) equipped with a laser-induced fluorescence (LIF) detector (P/ACE System Laser Module 488, excitation wavelength 488 nm). An RPR-100 photochemical reactor (Ultraviolet Company, Branford, CT, USA) was used for the photopolymerization reactions. A Shimadzu HPLC system (Kyoto, Japan) consisted of two LC-10AD pumps and an SCL-10A system controller. The analytical column was a Wakosil-II $3C_{18}$ RS (150 mm×4.6 mm I.D., 3-μm particle size, Wako) for reversed-phase chromatography. The column was maintained at 40 °C. A Hitachi F-1100 fluorescence spectrometer (Hitachi, Tokyo, Japan) was used for the detection of the derivatized amino acids (excita-

tion wavelength (λ_{ex}) 470 nm, emission wavelength (λ_{em}) 540 nm). The flow-rate was 1.0 ml/min.

2.3. Preparation of ODS monolithic column

The PSG monolithic column (total length 30 cm) was prepared from MPTMS using the same procedure described in our previous reports [30,31,36]. Scotch tape (Sumitomo 3M, Tokyo, Japan) was used to protect the UV-transparent capillary from the photo-induced polymerization reaction. The polymerization reaction took place only in the untaped section (13 cm) of the UV-transparent capillary. After drying the PSG monolithic column using an oven at 110 °C, the column was filled with a mixture of 4% DMOS and 8% diethylamine in toluene. Both ends of the column were sealed with Parafilm. The sealed capillary was kept at room temperature overnight. Finally, the capillary was washed with methanol to remove any unreacted reagents. Once fabricated, the ODS monolithic column (non-end-capped column) was conditioned with a mobile phase for approximately 5 min using a syringe and a hand-held vice. The column was further conditioned electrokinetically in the CE instrument by driving the mobile phase through the capillary at the applied voltage of 5 or 10 kV until a stable baseline was achieved.

2.4. Preparation of end-capped monolithic column

An ODS-modified PSG monolithic column was end-capped with CTMS. After drying the ODS monolithic column, it was filled with a mixture of 4% CTMS and 8% diethylamine in toluene and both ends of the column were sealed with Parafilm. The sealed capillary was keep at room temperature overnight. The capillary was washed with methanol to remove unreacted reagents and conditioned by the mobile phase as described above. In the subsequent text, we refer to this end-capped monolithic column as the end-capped monolithic column, whereas the ODS column, which was not end-capped, is referred to as the non-end-capped monolithic column.

2.5. Pretreatment of a CSF sample

A CSF sample was collected using microdialysis. This sample (20 μl) was filtered through a 0.2-μm filter (Millipore). The sample was derivatized with NBD-F following the procedure described below.

2.6. Derivatization reaction with NBD-F

Fig. 1 shows the scheme of NBD-F labeling of amino acids. A volume of 15 μl of 50 m*M* NBD-F in acetonitrile was added to the sample solution. The sample solution is a mixture of 25 μl of 50 m*M* borate buffer (pH 8.5) and 15 μl of either 100 m*M* amino acid solution or filtrate rat CSF. The NBD-F/sample mixture was mixed and heated at 60 °C for 5 min. A volume of 25 μl of mobile phase was added to the reacted solution, which was analyzed by CEC and HPLC. The sample was electrokinetically

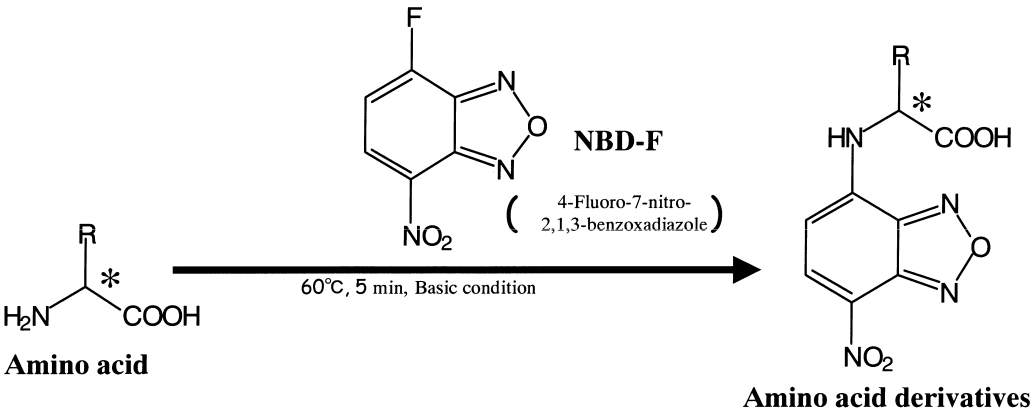

Fig. 1. Scheme of NBD-F labeling of amino acids.

injected into the capillary (5.0 kV or −5.0 kV, 5.0 s) for CEC.

2.7. Chromatographic parameters [37]

The exact calculation of the capacity factor *k* in CEC is very complicated because many factors must be determined. Therefore, in this study, retention times were used to examine the chromatographic performances of the modified PSG monolithic columns.

3. Results and discussion

In the analysis of charged analytes by CEC, two major driving forces work to cause migration. One is electroosmotic flow (EOF) and the other is the electrophoretic mobility of the analyte. In a fused-silica capillary, EOF is generated in neutral and basic conditions, but is negligible below pH 3. We examined elution time of thiourea, which was often used as an EOF marker [30,31]. Thiourea was eluted on both the non-end-capped and the end-capped monolithic column when using a mixture of ammonium acetate, water and acetonitrile. However, in the case of the mixture solution of phosphate buffer (pH 2.5), water, and acetonitrile, thiourea was not eluted. These results indicated that EOF was negligible on these modified PSG monolithic columns in acidic mobile phase condition. In this paper, we describe the elution behavior of NBD-derivatized amino acids under different mobile-phase conditions in a non-end-capped and an end-capped monolithic column.

3.1. Separation under neutral conditions

The separation of a test mixture of NBD-amino acids was carried out using a mobile phase composed of 50 m*M* ammonium acetate (pH 6.5)–water–acetonitrile. The effect of varying the water content in the range of 10–40% of the mobile phase was studied. No amino acids were eluted from either the non-end-capped or the end-capped monolithic column when the direction of EOF was from the inlet to the outlet. However, by reversing the applied voltage, such that the inlet is cathodic and the outlet is anodic, amino acids eluted on the end-capped monolithic column at almost the same time using a mobile phase with the composition 1:3:6 (v/v/v). The NBD-amino acids were eluted from the column by their electrophoretic velocities. On the other hand, no NBD-amino acids were eluted from the non-end-capped monolithic column under the same conditions. This behavior indicates that EOF is stronger in this column than for the end-capped monolithic column because of the presence of residual silanol groups in the non-end-capped monolithic column. The electrophoretic mobilities of the NBD-amino acids are the same on both monolithic columns.

NBD-amino acids were successfully eluted on a non-end-capped monolithic column when the water content in the mobile phase was reduced to 10% (i.e. buffer–water–acetonitrile, 1:1:8, v/v/v). We suggest that the reason for this behavior is a decrease in EOF or a change in the distribution ratio of the amino acids in the stationary phase and in the mobile phase [38]. NBD-Amino acids were also eluted on an end-capped monolithic column. Fig. 2 compares the elution times of the NBD-amino acids on both monolithic columns. A decrease in the water content of the mobile phase led to a decrease in the elution times of the NBD-amino acids compared to a mobile phase with higher water content (data not shown). The shorter elution times are ascribed to a higher distribution of NBD-amino acids in the mobile phase

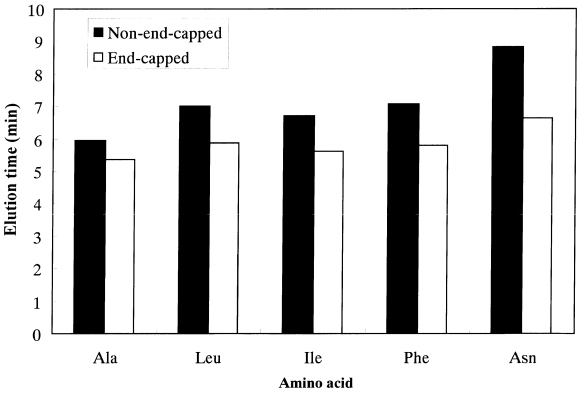

Fig. 2. Comparison of the elution times of NBD-amino acids on non-end-capped (■) and end-capped (□) column. Conditions: sample, 22 μ*M* NBD-amino acids; mobile phase, 50 m*M* ammonium acetate–water–acetonitrile (1:1:6, v/v/v); applied voltage, −10 kV; injection, −5 kV (5 s).

and a decrease in EOF velocity in the non-end-capped monolithic column. Furthermore, the elution time of each NBD-amino acid on the end-capped monolithic column was shorter than that on the non-end-capped monolithic column because of slower EOF in the end-capped monolithic column. We could not study the effect of higher water composition (≥40%) in the mobile phase because the amino acids were not eluted.

3.2. Separation under acidic conditions

Fig. 3 demonstrates the separation of five NBD-amino acids within 7 min on an end-capped monolithic column using a mobile phase containing 50 m*M* phosphate buffer (pH 2.5)–water–acetonitrile (1:1:8, v/v/v). Under these conditions, EOF was negligible, and the NBD-amino acids were eluted by electrophoresis at an applied voltage of −10 kV. The elution order of these NBD-amino acids was Asn, Phe, Ala, Ile, and Leu. Although Leu and Ile have the same molecular mass, these compounds were separated under these conditions. The theoretical plate numbers were 105 000, 58 700, 66 700, 62 200, and 63 500/m for Asn, Phe, Ala, Ile, and Leu, respectively. Compared to the end-capped monolithic column, the theoretical plate numbers of the NBD-amino acids on the non-end-capped monolithic column were smaller by 50–67%. The smaller theoretical plate numbers of NBD-amino acids is attributed to adsorption of the NBD-amino acids on the residual silanol groups on the non-end-capped monolithic column. Elution times of NBD-amino acids on the non-end-capped monolithic column were longer than those on the end-capped monolithic column. Separation of the NBD-amino acids under these conditions was not accomplished by free-solution capillary electrophoresis.

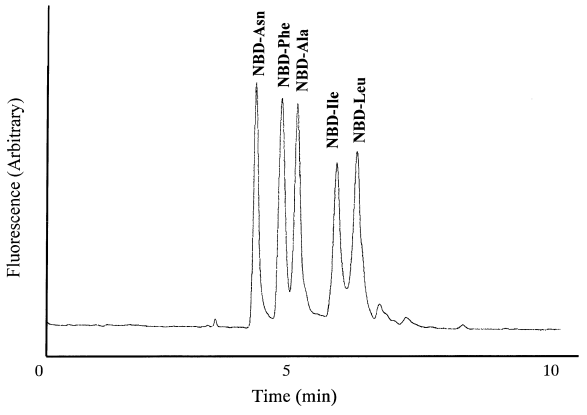

Fig. 3. Electrochromatogram of five NBD-amino acids. Conditions: sample, 22 μ*M* NBD-amino acids; mobile phase, 50 m*M* phosphate buffer (pH 2.5)–water–acetonitrile (1:1:8, v/v/v); capillary, end-capped column; applied voltage, −10 kV; injection, −5 kV (5 s); detection, excitation source is an argon ion laser.

The effect of water content in the mobile phase on the separation of the NBD-amino acids using the end-capped monolithic column was studied. The shortest elution times were achieved using a mobile phase with 30% water (i.e. buffer–water–acetonitrile, 1:3:6), but the resolution was poor. At 10% water in the mobile phase, the resolution was good although the elution times were slightly longer. Similarly longer elution times were also observed with 50% water content, but the resolution was inadequate. The elution time became longer when using higher water content and the solution also led to rapid peak broadening. A similar observation was reported by Hosoya et al. [39]. The use of mobile phase solutions with less than 10% water resulted in unstable baselines because of phosphate precipitation.

3.3. Analysis of rat CSF

The complexity of the rat CSF sample analysis is reduced because only NBD-amino acids are detected. (The derivatizing reagent, NBD-F, is not fluorescent.) Under our separation conditions, only the acidic compounds elute from the column, while the neutral and basic ones do not elute. Fig. 4 shows the electrochromatogram of a rat CSF sample using an end-capped monolithic column with a mobile phase of 50 m*M* phosphate buffer (pH 2.5)–water–acetonitrile (1:1:8, v/v/v). Both Ser (5.18 min) and Gln (5.70 min) were detected under 6 min. CSF amino acids were identified on the basis of spiking experiments with standard amino acids. These two amino acids were also detected by HPLC with fluorescence detection, but it was hard to determine each amino acid quantitatively because of its low concentration. Furthermore, the retention times (30 min) were longer than those in CEC. Others [33–35] have detected these amino acids in rat CSF. The detector

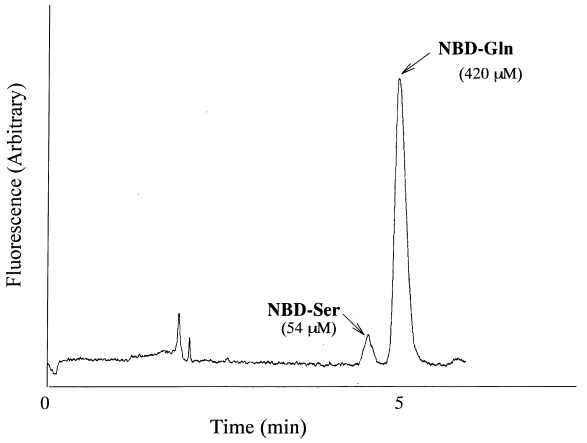

Fig. 4. Electrochromatogram of a rat CSF sample. Separation conditions are the same as in Fig. 3.

output over a concentration range of 0.025–2 μ*M* was linear for Gln (R^2=0.998) and Ser (R^2=0.996). The concentration of Gln and Ser measured was 420 and 54 μ*M*, respectively, which is similar to the data reported for rat CSF (549 and 75 μ*M*, respectively) [33]. Other peaks in Fig. 4 were not identified, and they may be some byproducts of the derivatization reaction. The run-to-run repeatability of the same monolithic column in this separation condition was measured. The RSD of the elution time was less than 0.5% (n=3). The batch-to-batch reproducibility of the non-end-capped monolithic column was also acceptable and the RSD of the elution time was less than 5% (n=3).

4. Conclusion

A non-end-capped and an end-capped ODS-modified PSG monolithic column were prepared for the separation of NBD-amino acids. The end-capped PSG monolithic column was shown to be superior to the non-end-capped monolithic column for the separation of NBD-amino acids. CEC with LIF detection and an end-capped monolithic column allowed for simpler sample pretreatment of low volumes of rat CSF. Rapid separation of the amino acids in CSF was possible.

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