Confirmation of Separation Mechanism Through Visualization of Microfluidic Behavior of Fluorescent Analytes in Tube Radial Distribution Chromatography

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Abstract
A method of tube radial distribution chromatography (TRDC) based on an annual flow with inner and outer phases created through phase transformation in an open-tubular capillary was developed. The outer phase works as a pseudo-stationary phase under laminar flow conditions in TRDC. Two model fluorescent analytes, hydrophobic perylene and hydrophilic Eosin Y, were separated by TRDC using a water/acetonitrile/ethyl acetate mixed solution (3:8:4 volume ratio, organic solvent-rich or 4:3:1 volume ratio, water-rich) as an eluent solution and a fused-silica capillary tube (75 μm inner diameter and 120 cm length/100 cm effective length) as a separation column. We observed for the first time microfluidic behavior of fluorescent analytes that distributed and separated in the capillary tube visually by fluorescence microscope-charge coupled device camera. The separation mechanism in the TRDC was confirmed with visual fluorescence data and the obtained chromatographic data with UV detection. Furthermore, the elution times of perylene and Eosin Y were calculated based on their retention factors for the two phases and laminar flow conditions. The data and results obtained support the proposed separation mechanism in TRDC.

Keywords: Tube radial distribution chromatography; Separation mechanism; Visualization; Fluorescent analyte

1. Introduction
Capillary chromatography, in particular using an open-tubular capillary, is a unique and remarkable separation method that features small solvent and sample volume, simple apparatus, easy operation, and low cost [1,2]. However, few novel techniques regarding open-tubular capillary chromatography have been studied in this century. One example, wide-bore hydrodynamic chromatography [3,4] was reported using an open-capillary tube of fused silica. Non-diffusive and diffusive analytes indicated markedly different elution behavior in the capillary tube under laminar flow conditions. Another example, micellar electrokinetic capillary chromatography, is a separation mode used in capillary electrophoresis, where the micelle works as a pseudo-stationary phase during chromatographic separation [5,6].

In contrast, we have reported that phase-separation multiphase flow is generated with a two-phase separation mixture, such as a ternary mixed solution of water/acetonitrile/ethyl acetate, through phase transformation via change in temperature and/or pressure [7,8]. The two-phase mixed solution changes from homogeneous to heterogeneous with upper and lower phases in a batch vessel through phase transformation.
However, when the mixed solution is fed into a micro-space, such as a capillary tube, changes in temperature and/or pressure results in phase-separation multiphase flow, through a kinetic liquid-liquid interface. Under certain conditions, annular flow consisting of inner and outer phases can be observed as a phase-separation multiphase flow, which we call tube radial distribution flow (TRDF). Novel open-tubular capillary chromatography has been performed based on specific TRDF, which we name separation method tube radial distribution chromatography (TRDC) [9,10]. The outer phase in TRDF acts as a pseudo-stationary phase under laminar flow conditions during chromatographic separation.

To date, perylene and Eosin Y have been dissolved in the water/acetonitrile/ethyl acetate mixed solution to form an eluent, and observed by fluorescence microscope-charge coupled device (CCD) camera in order to confirm TRDF formation with inner and outer phases in a capillary tube. In this study, for the first time perylene and Eosin Y were introduced into a capillary tube via gravity as an analyte in TRDC, previously only used as analytes in micro-channel TRDC on a micro-chip [11]. The microfluidic behavior of the analytes distributed and separated in the capillary tube was directly and visually observed by fluorescence microscope-CCD camera. In addition, they were chromatographically separated and detected through TRDC equipped with a UV detector. The separation mechanism in TRDC was analyzed and confirmed with the new data obtained in this study.

2. Experimental

2.1. Reagents and materials

Purified water was obtained from an Elix 3 UV water purification system (Merck, Darmstadt, Germany). All reagents were commercially available and of analytical grade. Perylene, Eosin Y, acetonitrile, and ethyl acetate were purchased from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). Untreated- and inert-inner wall fused-silica capillary tubes with of inner diameter 75 µm and outer diameter 150 µm were purchased from GL Science Co. (Tokyo, Japan). These were used for the organic solvent-rich and water-rich eluent solutions of mixed water/acetonitrile/ethyl acetate, respectively [7,8].

2.2. Flow system equipped with fluorescence microscope-CCD camera

A schematic diagram of the flow system equipped with the fluorescence microscope-CCD camera is shown in Fig. 1(A), comprising of a fused-silica capillary tube (120 cm length) and microsyringe pump (MF-9090; Bioanalytical Systems, Inc., IN, USA). The tube temperature was controlled with a thermo plate (MAT5S-555S; Tokai Hit Co. Ltd., Shizuoka, Japan). The fluorescence in the capillary tube was monitored at approximately 100 cm from the capillary inlet using a fluorescence microscope (BX51; Olympus, Tokyo, Japan) with an Hg lamp, optical filters (U-MWU2; 330–385-nm excitation filter and >420 nm emission filter), and CCD camera (JK-TU53H; Toshiba, Tokyo, Japan). The obtained fluorescent photograph data was exported to computer for drawing fluorescent profiles. The water/acetonitrile/ethyl acetate mixed solution was employed as the eluent, and used to prepare the fluorescent analyte solution including 0.1 mM perylene and 1.0 mM Eosin Y.

The fluorescent analyte solution was directly introduced into the capillary inlet side via gravity (30 cm in height × 15 s, ca. 30 nL). After analyte injection, the capillary inlet was connected through a joint to the microsyringe, which was combined with a pump in order to feed the eluent solution into the capillary tube at a fixed flow rate (0.8 µL/min) under laminar flow conditions. Two types of water/acetonitrile/ethyl acetate mixed solutions were prepared as elutions; an organic solvent-rich, 3:8:4 volume ratio and a water-rich, 4:3:1 volume ratio. The fluorescent photographs of analytes, perylene (blue) and Eosin Y (green), were visually observed by the flow system equipped with a fluorescence microscope-CCD camera.

When observing the TRDF image in the capillary tube, perylene and Eosin Y were wholly dissolved in the water/acetonitrile/ethyl acetate mixed solution to give an eluent containing 0.1 mM perylene and 1.0 mM Eosin Y. The eluent containing fluorescent compounds was continuously delivered into the capillary tube.

2.3. TRDC system

The TRDC system was similarly incorporated into the flow system. A schematic diagram of the TRDC system comprising of a fused-silica capillary (120 cm length/100
cm effective length), microsyringe pump, and UV detector (SPD-20AV spectrophotometric detector (Shimadzu Corporation, Kyoto, Japan) was modified to on-capillary detection) is shown in Fig. 1(B). The tube temperature was controlled by submerging the capillary tube into a beaker of water maintained at a fixed temperature by stirring. The two types of eluent solutions, organic solvent-rich and water-rich, as well as the fluorescent analyte solution including 0.1 mM perylene and 1.0 mM Eosin Y were prepared as mentioned above.

The analyte solution was directly introduced into the capillary inlet side by gravity. The eluent solution was fed into the capillary tube at a fixed flow rate (0.8 μL/min) under laminar flow conditions. On-capillary UV detection (254 nm) was performed using the spectrophotometric detector to generate the chromatograms.

3. Results and discussion
3.1. TRDF creation with organic solvent-rich and water-rich eluent solution

TRDF formation was confirmed at 5°C from delivery of the water/acetonitrile/ethyl acetate mixed solution into the flow system. Fluorescence photographs of TRDF are shown in Fig. 2 together with the phase diagram of the ternary mixed solution of water/acetonitrile/ethyl acetate. The dotted lines in the diagram are the solubility curves between heterogeneous and homogeneous composition areas at 5 and 25°C, respectively.

By cooling the eluent from 25 to 5°C, in the organic solvent-rich eluent solution we could observe the organic solvent-rich major inner phase, where hydrophobic perylene (blue) was distributed, and the water-rich minor outer phase, where hydrophilic Eosin Y (green) was distributed. In contrast, in the water-rich eluent solution we could observe the water-rich major inner phase, where hydrophilic Eosin Y (green) was distributed, and the organic solvent-rich minor outer phase, where hydrophobic perylene (blue) was distributed. We observed the reverse configuration pattern in TRDF between the organic solvent-rich and water-rich eluent solution. The specific reverse microfluidic behavior was analyzed and examined using the viscous dissipation principle and linear stability analysis [12]. As expected, TRDF did not occur at 25°C for the organic solvent-rich and water-rich eluent solution, where a homogeneous image, i.e. non-TRDF, was observed as shown in Fig. 2.

3.2. Fluorescence observation and chromatogram of fluorescent analytes with organic solvent-rich eluent solution

Delivery of the organic solvent-rich solution into the capillary tube confirmed TRDF with an organic solvent-rich major inner phase and water-rich minor outer phase (Fig. 2). Microfluidic behavior of the fluorescent analytes was visually observed using the organic solvent-rich solution as an eluent through the flow system (Fig. 1(A)). Figure 3 shows the microfluidic behavior of the model analytes, hydrophobic perylene (blue) and hydrophilic Eosin Y (green), that were injected into the capillary tube via gravity. Hydrophobic perylene was distributed in the inner phase and hydrophilic Eosin Y in the outer phase. Perylene was faster than Eosin Y under laminar flow conditions when...
using the organic solvent-rich solution, where the water-rich minor outer phase worked as a pseudo-stationary phase. Visual separation behavior of the analytes in the capillary tube was observed, confirming the separation mechanism in the TRDC.

In addition, we examined the analytes, perylene and Eosin Y, using the TRDC system (Fig. 1(B)) with the organic solvent-rich eluent solution. The obtained chromatogram in Fig. 4(A) indicates they were successfully separated each other. The elution times were 5.8 and 7.0 min for perylene and Eosin Y, respectively.

3.3. Fluorescence observation and chromatogram of fluorescent analytes with water-rich eluent solution

During delivery of the water-rich solution into the capillary tube, TRDF with a water-rich major inner phase and organic solvent-rich minor outer phase was confirmed (Fig. 2). Microfluidic behavior of the fluorescent analytes was visually observed using the water-rich solution as an eluent through the flow system (Fig. 1(A)).Figure 5 shows the microfluidic behavior of the model analytes, hydrophobic perylene (blue) and hydrophilic Eosin Y (green), injected via gravity. Hydrophilic Eosin Y was distributed in the inner phase and hydrophobic perylene in the outer phase. Eosin Y was faster than perylene under laminar flow conditions using the water-rich solution, where the organic solvent-rich minor outer phase worked as a pseudo-stationary phase. Visual separation behavior of analytes in the capillary tube was also observed, confirming the separation mechanism in TRDC with the water-rich solution.

We examined the analytes of perylene and Eosin Y through the TRDC system (Fig. 1(B)) with the water-rich solution. The obtained chromatogram Fig. 4(B) indicates they were successfully separated from each other. The elution times were 5.5 and 6.8 min for Eosin Y and perylene, respectively. We were able to observe the reverse elution order of fluorescent analytes with both organic solvent- and water-rich solutions in the fluorescent photographs (Figs. 3 and 5) and chromatograms (Fig. 4).

But the chromatogram Fig. 4(B) obtained with the water-rich solution showed unstable peak shape. The reason has not been clear yet. For the moment, we think that the instability may be caused by unstable flow behavior of the organic solvent-rich minor outer phase on the inner wall of fused-silica capillary tube, although it was improved by using an inert-inner wall fused-silica capillary tube instead of an untreated-wall one. The reproducibility of peak heights and areas for TRDC with the organic solvent-rich solution was reported in the previous paper [13]. These data for TRDC with the water-rich solution will be examined, making technical improvements.

3.4. Estimation of elution times for analytes

The distribution coefficient $K_d$ of perylene and Eosin Y in the upper (organic solvent-rich) and lower (water-rich) phases generated through phase transformation of the
ternary solvent in the batch vessel was investigated. The analyte concentrations in each phase were estimated by absorption measurements and calibration data.

The calculation of the retention factor $k'$ from the $K_d$ value was easily carried out through the relation:

$$k' = \frac{(C_sV_s)}{(C_mV_m)} = K_d \frac{(V_s)}{(V_m)},$$

(1)

where $C$ and $V$ refer to the analyte concentration and phase volume, respectively, and subscripts $s$ and $m$ refer to the stationary and mobile phases, respectively. The $K_d$ and $k'$ values of the upper (organic solvent-rich) and lower (water-rich) phases were determined for the analytes, perylene and Eosin Y, using the ternary mixed solution of water/acetonitrile/ethyl acetate. The volume ratios of the upper and lower phases in the batch vessel were 4:1 for the organic solvent-rich eluent solution (water/acetonitrile/ethyl acetate, 3:8:4, volume ratio) and 1:5 for the water-rich eluent solution (water/acetonitrile/ethyl acetate, 4:3:1 volume ratio), respectively. The $K_d$ and $k'$ values of the organic solvent-rich eluent solution were 4.2 and 1.1 for perylene and 10 and 2.5 for Eosin Y, respectively. For the water-rich eluent solution the values were 7.1 and 1.4 for perylene as well as 3.0 and 0.59 for Eosin Y, respectively.

The theoretical elution times of the TRDC system were determined assuming that the stationary phase is not static and flows slowly relative to the mobile phase, according to a parabolic curve of linear velocity under laminar flow conditions. The parabolic curves for the organic solvent-rich and water-rich eluent solution can be drawn [10,12] under the present conditions as shown in Fig. 6. Hence, the elution time $t_R$ could be calculated based on the values of $k'$, $t_m$, and $t_s$ using Eq. (2) and taking advantage of the parabolic curves [10]:

$$t_R = t_m \frac{(k'+1)}{(1 + k't_m/t_s)},$$

(2)

If an analyte is completely distributed in the inner phase of the TRDF the elution time from the capillary inlet to detector is $t_m$, and if completely distributed in the outer phase, $t_s$. The theoretical elution times in TRDC were estimated using a previously developed method [10] and Eq. (2). According to theory, perylene and Eosin Y are eluted in 5.6 and 6.8 min for organic solvent-rich eluent solution and 6.4 and 5.3 min for water-rich eluent solution, respectively. The experimental and theoretical elution times were relatively consistent with each other. The analysis of elution time supports the chromatographic observation of separation in TRDC based on analyte distribution between an inner (mobile) and outer (pseudo-stationary) phases in a capillary tube.

4. Conclusion

Previously, perylene and Eosin Y dissolved in water/acetonitrile/ethyl acetate mixed solution as an eluent had been observed by fluorescence microscope-CCD camera in order to detect TRDF formation with inner and outer phases in a capillary tube. In this study, perylene and Eosin Y were introduced into TRDF for the first time as a model analyte in order to directly observe their micro-fluidic behavior. The separation mechanism in TRDC was confirmed in visual fluorescence data and the obtained chromatographic data with UV detection.

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