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Direct observation of the separation process by the chromato-videoscope[1]

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Abstract

Separation processes in a liquid chromatographic column were visualized and analyzed by a developed chromato-videoscope. The migration aspects were evaluated with successively obtained densitograms. In reversed-phase chromatography, the band widths of each solute band were almost equal fore both weakly and strongly retained solutes when compared at the same column position (the same migration distance in the column). In the gradient elution mode, the position of the solute band showed that the migration velocity of the solute band changed gradually according to changes in solvent composition. The drug trapping process to BSA- coated ODS packings for direct injection of biological fluid was also observed. In the absence of BSA from the sample solution, the drug molecules were trapped in a narrow band. However, at higher BSA concentrations in the sample solution, a broader band shape was observed. This band broadening shows how the drug molecules were retained on the protein. © 1997 Elsevier Science B.V.

Keywords: HPLC; Gradient method; Direct injection method; Separation mechanism; Video analysis; Image analysis

1. Introduction

In general, the performance of liquid chromatography has been assessed with a chromatogram which shows the changes in the solute band concentration at a detecting point where solutes were eluted from the column. However, it seems necessary to observe and analyze the separating process of the solute band when it is in the column, since the column is where separation of solutes is taking place. A few attempts were investigated to observe directly the behaviour of solutes band in the chromatographic column. Bussolo et al. discussed the visualization of protein retention and migration in reversed phase liquid chromatography [1]. Ilg et al. tried to observe the band profiles in a preparative column by magnetic resonance imaging [2].

On the other hand, multichannel detection has became widely used in capillary electrophoresis (CE) for direct observation of the dynamic aspects of solutes in a capillary. Cheng et al. used a charge coupled device as the fluorescence detector

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for CE to obtain a three-dimensional electropherogram [3]. Tsuda et al. [4], Taylor et al., [5] and Razee et al., [6] have discussed migration aspects in CE using video detection. Sweedler et al. utilized the time-delayed integration mode to detect a fluorescein isothiocyanate-labelled amino acid in the 10–20 mole range [7]. Furthermore, multi-channel detection has been applied to various spectroscopies and separation sciences [8–12].

A method was developed for direct observation of the dynamic aspects of solute band movements in a liquid chromatographic column using video image analysis [13–15]. The separation of solute bands was successively monitored by a video camera and each video image was computer-processed to produce a densitogram which shows the distribution of the solutes along the central line of a column at a certain time. Various unknown aspects were clarified by this method especially in the stepwise elution mode [15]. In this report, the behaviour of the solute bands has been investigated in other separation and trapping modes.

2. Experimental

2.1. Chemicals

Dansyl alanine (DNS-Ala), dansyl glycine (DNS-Gly), dansyl leucine (DNS-Leu) and dansyl valine (DNS-Val) were purchased from Seikagaku Kogyo (Tokyo, Japan). Bovine serum albumin (BSA) was purchased from Sigma. Solvent was HPLC-grade acetonitrile. A Mili-Q system was used for water purification. All other chemicals were of reagent-grade quality and used without any further purification.

2.2. Apparatus

The HPLC apparatus consisted of two HPLC pumps (880-PU, JASCO, Japan), an injection valve (7125, Rheodyne, USA), and a glass column (Pharmacia, Sweden). A video image which was obtained by a video camera with three charge-coupled devices (DXC-930, Sony, Japan) was recorded using a standard video tape recorder (AG-7355, Panasonic, Japan) or a time-lapse video tape recorder (AG-6720A, Panasonic, Japan). The analyzing system of the video image consists of an image processor (PIP-4000, ADS., Japan) connected with a microcomputer (PC-98, NEC, Japan) for ordering commands for analysis. Further calculation and simulations were performed with a Power Macintosh 8100/80 AV computer (Apple Computer, USA).

3. Chromatographic conditions

3.1. Isocratic elution mode

A 115 × 5 mm i.d. column was packed with 9 μm Chemcosorb ODS-H (Chemco, Japan) in our laboratory. The mobile phase was 0.1 M acetic acid-acetonitrile (80:20, v/v). The sample solution was prepared with the mobile phase.

3.2. Gradient elution mode

The same column was used. The composition of the mobile phase from 0–3 min was 0.1 M acetic acid–acetonitrile (80:20, v/v); from 3–6 min the composition was changed under linear gradient conditions to 0.1 M acetic acid–acetonitrile (60:40, v/v) 6 min and was then kept constant.

3.3. Direct injection study of protein containing samples onto BSA-coated ODS column

A 65 × 5 mm i.d. glass column was packed with 30 μm Chemcosorb ODS 30–120 (Chemco, Japan) in our laboratory. Before the sample injection studies, 3% BSA in phosphate buffered saline (PBS, pH 7.4) was injected on to the column with the PBS mobile phase until BSA adsorbed on the surfaces of ODS resin attained saturation [16]. Then more than three times the amount of the column volume of methanol was introduced into the column in order to desorb the BSA from the outer surface of the ODS packing [16]. These procedures were repeated at least thrice. Sample solutions were prepared with PBS or BSA in PBS solution.

3.4. Detection and analysis method

Fluorescence images of DNS-amino acid bands (Excitation 340 nm, Emission 550 nm) along the column were monitored by the video camera under a Black Ray lamp illuminator (UVC, USA). An optical filter (SC-42, Fuji-Film, Japan) was placed to cut off the excitation light. Fluorescence intensities at each point (pixel) in the image were converted into digital data by an image processor and used for analysis. The densitogram was obtained by plotting the value of fluorescence intensities along the centre line of the column.

4. Results

4.1. Isocratic elution mode

Fig. 1 shows the relationship between band width of solutes and the migration distance of the solute band. The band widths increased according to the migration distances, however, the extents of band broadening were almost the same at the same migration distance in a column. It might be though that a solute which migrates fast passes through the column with a narrow distribution, whereas the band width of a solute which migrates slowly, becomes broader. In this experiment, the band widths of all solutes at the same point in the column are almost the same within a 10–15% evaluation error even though DNS-Leu is highly lipophilic DNS-Gly is less lipophilic. This result shows that the numbers of theoretical

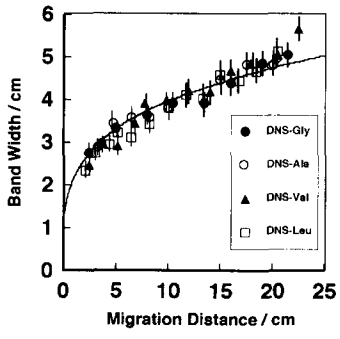

Fig. 1. Band widths of four DNS amino acids at various points of migration on a HPLC column.

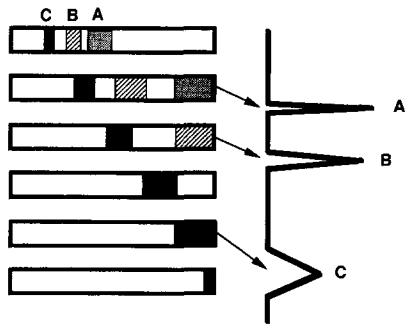

Fig. 2. Scheme of band broadening of various separating solutes on migration in the column in comparison with ordinary chromatogram (right side).

plates of both DNS amino acids are almost the same under these typical partition reversed-phase conditions. As illustrated in Fig. 2, it appears that both fast migrating solute A and slow migrating solute C show almost the same band width at the same position in the column. thus, differences in the peak width of each sample in the ordinary chromatograms are due to differences in the resident time in the column. In other words, solute A in Fig. 2 requires less mobile phase to be eluted, but solute C needs much more mobile phase to be eluted from the bottom of the column.

5. Gradient methods

The present method was applied to observe the positions of four dansyl amino acids bands in a column under the gradient elution mode. Fig. 3 shows a contour map which was constructed by accumulation of successive densitograms obtained by the present method. Each band shows a straight part where the solute migrates with constant velocity by the first solvent, a curved part where the solute migrates with varied velocity by mixing of the first solvent and the second solvent and another straight part where the solute migrates by the second solvent.

Fig. 4 illustrates a zone model in order to explain the migration profile. The migration profile was divided into three zones which were separated by two straight lines. These straight lines show the movement of the solvent front

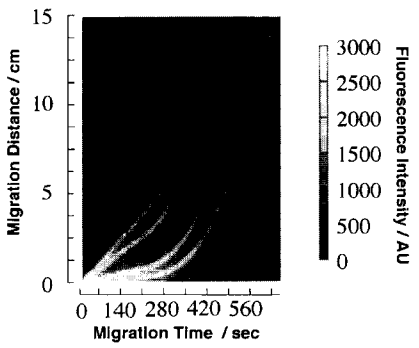

Fig. 3. Three dimensional migration profiles of four DNS amino acids (four trails in this graph. DNS-Gly, -Ala, -Val and -Leu from left–right) in the gradient elution mode. The Z axis is the fluorescence intensity of each DNS amino acid band. Separation proceeds from left to right along the time axis.

[14,15]. In the first zone, the solutes migrates by the first solvent and the migration distance (d) is simply described as:

$$d = K_1 \times t$$

where K_1 is the velocity constant of the first solvent, t is the migration time and t_1 is the time when the migration velocity starts to change. The second zone containing curved parts is described as:

$$d = K_1 \times t_1 + f(t)\, dt$$

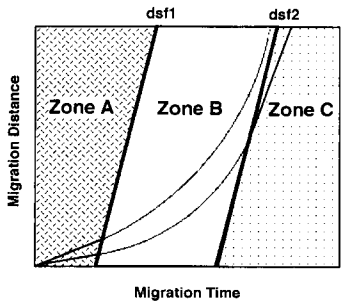

Fig. 4. Schematic partition of migration profiles of Fig. 3 into the first and last isocratic elution zone and the centre gradient elution zone. The left solid line (with dfs1 sign) shows the process of ending the first isocratic elution and the right solid line (with dfs2 sign) shows the process of starting the last isocratic elution with the final eluent.

where $f(t)$ is the migration velocity which is a function of time, t_2 is the time when the solute band completely migrates at a particular velocity and then t_2 becomes constant.

$$d = K_1 \times t_1 + f(t)dt + K_2 \times (t_2 - t)$$

Where u is the linear velocity of the solvent, the demixing solvent fronts, dsf_1 and dsf_2, which are shown as bold lines (A) and (B) in Fig. 4, are described, respectively, as:

$$d_{sf1} = u \times (t - t_{c1})$$

and

$$d_{sf2} = u \times (t - t_{c2})$$

By rearrangement of these equations, the position of the solute band can be described. There have been many investigations to simulate the band migration profiles in the gradient elution mode [17,18]. It is important to predict the retention time of the solute band in order to specify acceptable gradient conditions. However, these approaches are essentially empirical and the mechanism of the migration process has been speculation rather than known by chromatographers. The difficulties of simulation in the gradient mode meant that the $f(t)$ function could bot be defined. The proposed method could provide useful information about $f(t)$. Since it is not enough to stimulate at the migration profile completely further information has to be given for the appropriate setting of gradient conditions.

6. Direct injection study of protein-containing sample onto a BSA coated ODS column

For the determination of the drug concentration in biological fluids, surface-inert reversed-phase packing was used as a pretreatment column for the direct injection of biological fluids [15,19–23]. Fig. 5 shows the densitograms 1 min after injection when a DNS-Gly solution sample containing BSA was injected on to the BSA-coated ODS column [20,24] which is a surface-inert reversed-phase column. The solute band was formed at the top of the column and the band width of trapped DNS-Gly increased according to

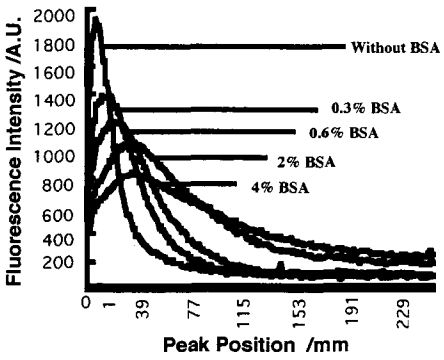

Fig. 5. Comparison of densitograms of the column trapping profiles of DNS-Gly at the top range of a protein-coated ODS column. The contents of BSA in the sample solutions are shown in the figure.

the increase in BSA concentration. The band shape did not change once it had been formed because the mobile phase contained no organic solvent. Thus, the difference in the trapped band shape depends on the injected solvent. It is believed that highly water soluble compounds and large-molecular compounds such as proteins are eluted out but lipophilic drugs are retained on the stationary ODS phase in the column.

According to this assumption, the result obtained could be explained by postulating that free DNS-Gly molecules are immediately trapped into the pores of the BSA-coated ODS packing whereas BSA, which comprises water-soluble large molecules, migrates and reacts with DNS-Gly released from BSA is trapped onto the stationary phase. As a result of repetition of these processes, solutes are dispersed along the column and form a broad band. Since the number of DNS-Gly molecules bound to BSA increases in proportion to the BSA concentration in the sample solution, the band width becomes broader according to an increase in BSA concentration. Shibukawa et al. discussed phenomena at the top of the column using high-performance frontal analysis which is one of the behaviour of the solute not only at the top of the column but also over the whole column length [25]. The proposed method represents one approach to observe and analyze solute behaviour in the whole column.

Fig. 6. shows the relationship between the concentration of BSA in the injection solvent and the band width of four DNS-amino acids. The extent of change in the trapped band width varied between DNS-amino acids. Thus, the extent of widening of the solute band was compared with the binding constant between BSA and each DNS-amino acid. The band width of strongly binding solutes tended to become wide. It seems that this difference in the band width of these samples is mainly due to the difference in the strength of protein binding.

It was found also that the injection volume affects the trapped band shape [26]. In the absence of BSA, the band shapes were almost similar to each other even if the injection volume was different. However, in the presence of BSA, band shapes become broad according to the increase in injection volume. It appears that once the BSA releases the DNS-amino acid by trapping on the solid phase then free BSA reacts with part of the amino-acid from the solid phase during migration.

When extra tryptophan was added to the sample solution, the band width of DNS-Gly became narrow depending on the amount of added tryptophan, as shown in Fig. 7. This is explained by postulating that binding sites of DNS-Gly to BSA compete with tryptophan in the sample solution and that there was an increase in free DNS-Gly. the present method can be applied not only to the

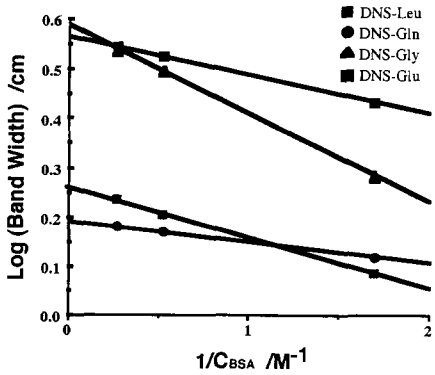

Fig. 6. Relationship between the logarithm of the trapped band width of DNS-Gly which is shown in Fig. 5 and the reciprocals of BSA concentration in the sample solutions.

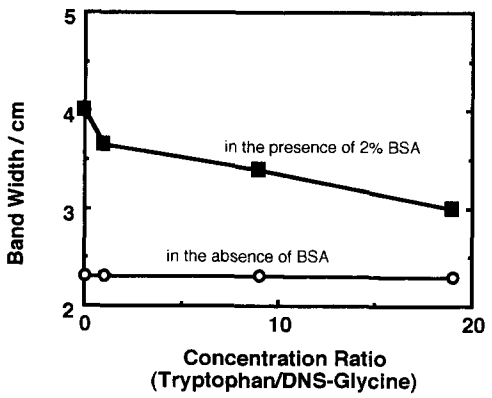

Fig. 7. Effect of tryptophan added tot he sample solution on the band width of trapped DNS-Gly as shown in Fig. 5.

separation mechanism but also to the analysis of protein binding sites.

Some potential value of this direct observation method to investigate the separation mechanism has been shown. It is only one facet of the performance of the whole column detection method. The importance of the analysis of solute behaviour in the column has been recognized. Gelderloos et al. discussed the utility of whole column detection using computer simulation [27]. Because of difficulties in detection, however, it has been left out of the present investigation. The present method provides one technique for direct imaging of separation processes in liquid chromatography.

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