Direct Observation of Solutes Trapping on a Surface-inert Reversed Phase Column

Atsushi Tamura, Maasoomeh Khademizadeh, Saeid Razee, and Tsutomu Masujima*
Institute of Pharmaceutical Sciences, Hiroshima University, School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima 734

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The solute trapping process onto the surface-inert reversed phase packing was directly investigated with chromato-videoscope. The Fluorescence intensity of separating dansyl (DNS) amino acids in the column was detected by video camera and densitogarm of solutes along the column length was obtained with computer-processing of the video images. Band widths of trapped DNS amino acids were narrow in the absence of BSA in sample solution. However, in the presence of BSA, band shapes turned broad according to increase of the concentration of BSA or injected volume of the sample.

Sample pre-treatments, e.g. de-proteinization and pre-concentration, are important when we determine a drug-level concentration in biological fluids. Surface-inert reversed phase packing is used as pre-treatment column for direct injection of biological fluids since the surface of the packing has no interaction with serum proteins but can trap the lipophilic drugs into the pores.[1-5] However, in the presence of proteins in sample solution, the trapping process of drug and re-migration process after solvent switching has been under imagination. We had developed the chromato-videoscope which is able to directly observe and analyze the solutes band during migration in column.[6] In this study, the direct observation of DNS-amino acids trapping onto the BSA-coated ODS column which was first packing of surface-inert reversed phase column[7] was investigated.

Mobile phase of trapping procedure was an aqueous solution without organic solvent to prevent protein denaturation. By this solvent, the proteins are washed out but lipophilic drugs are trapped into the pore of packing. Figure 1 shows the video images of trapped DNS-glycine (Gly) bands in the absence and presence of 2% BSA. In the absence of BSA, the trapped band was sharp at the top of column but became broad in the presence of BSA. It seemed that free DNS-Gly molecules were trapped into the pore of packing immediately but BSA bound DNS-Gly migrated with BSA. However, after slight migration, some part of bound DNS-Gly were released from BSA in order to achieve an equilibrium state and then released DNS-Gly from BSA are trapped onto the pores of column. As a result of repetition of this process, solutes were dispersed along the column as shown in Figure 1.

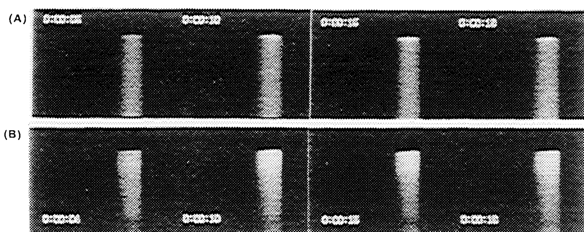

Figure 1. Video image of trapped DNS-Glycine (injection volume of 50μl), (A)in the absence of BSA, and (B)in the presence of 2% BSA. Mobile phase was 0.1M phosphate buffer (pH=7.4) at flow rate of 2 ml/min.

A densitogram along the column length which shows the solute's fluorescence intensity as a function of distance from inlet of column is obtained by the image analysis of video images. Figure 2 shows the densitograms with various concentration of BSA in sample solution. When BSA concentration was increased, the band shapes turned lower and broader owing to the high proportion of bound DNS-Glys to BSA. Furthermore, in Figure 2 the band peaks shifted to far side from top of the column according to an increase of BSA concentration. The reason is presumed that when injected solution contains BSA, free DNS-Gly can not easily move into the pores. As shown in Fgure 3, the injection volume also affects the trapped band shape. In the absence of BSA, the band shapes were almost similar even if injection volume was different. However in the presence of BSA, band shapes become broad according to the increase of injection volume. This fact supports that the band broadening is caused by protein binding. The extent of band broadening seemed to reflect the binding strength between proteins and DNS-Glys. Shibukawa et al. were introduced a high performance frontal analysis (HPFA) which is one of the useful determination methods of protein binding constant.[8] The present method is also available to estimate binding constant with less amount of injection volume.

From these results, it was supposed that the band shape was subjected to change by artificial manipulation of bound/free ratio if the extent of band broadening depended on protein binding. When we added extra tryptophan(Trp), to the sample solution, the band shape of DNS-Gly became narrow. This is explained that binding site of DNS-Gly to BSA was competed by Trp. Thus, the present method can also be applied for the binding

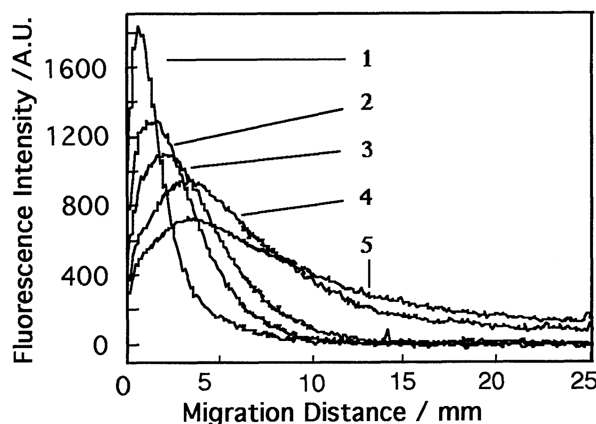

Figure 2. Effect of concentration of BSA on the band shape of DNS-Gly (injection volume = 50μl). BSA concentration were (1)0, (2) 0.3, (3)0.6, (4)2 and (5)4%, respectively, flow rate of 2 ml/min.

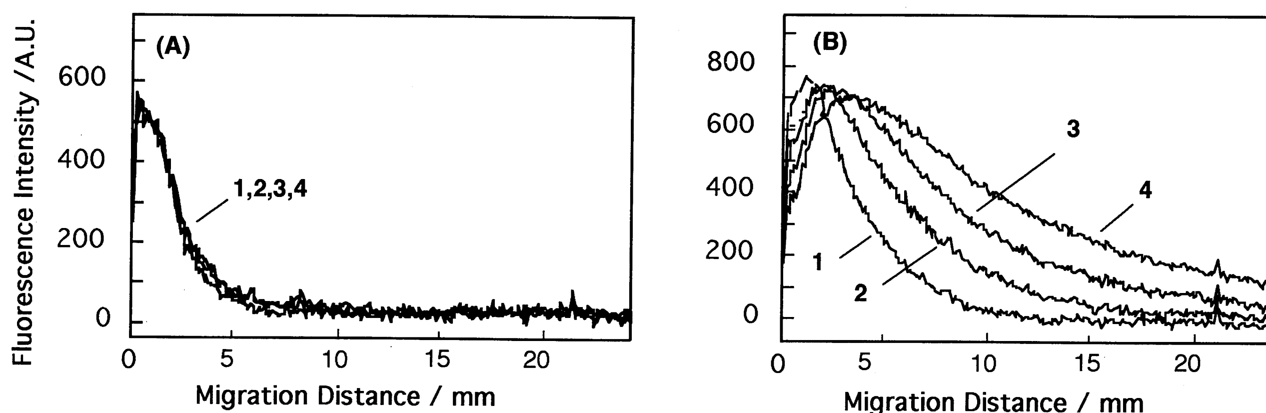

Figure 3. Effect of injection volume on the band shape of DNS-Gly. (A)In the absence of BSA, and (B)With 2% BSA at injection volume of (1)10μl, (2) 20μl, (3)50μl, (4)100μl, respectively.

competition studies.

Direct observation of solutes behavior in trapping step, was investigated on the BSA-coated ODS column in the presence of proteins in sample solution. The present method can show the ability for not only LC direct detection method but also analyzing tools for multiple equilibrium phenomena, such as partition and protein-drug binding. We are studying the applicability of this method to investigate the interaction of drugs with proteins, enzymes, as well as receptors.

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