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Diol-bonded silica gel as a restricted access packing forming a binary-layered phase for direct injection of serum for the determination of drugs

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Abstract

Direct serum injection for drug determinations can be achieved on a diol-bonded silica gel as a restricted access packing. The diol-bonded phase, 3-(2,3-dihydroxypropoxy)propylsilylsilica, contains two different functions, a hydrophilic function at the tip of the single chemical bond and a hydrophobic function on the inside part of the bond to form a "binary-layered phase" on the support surface. Proteins, as large molecules, contact only the hydrophilic surface of the diol phase, and they are eluted at the solvent front based on size-exclusion chromatography. On the other hand, small molecules such as synthetic drugs are retained on the internal hydrophobic function and separate based on reversed-phase chromatography. Accordingly, the diol-bonded silica gel performs as a restricted access packing for direct serum injection for the determination of relatively hydrophobic drugs.

1. Introduction

There have been a number of investigations concerned with protein separations by reversed-phase high-performance liquid chromatography (RP-HPLC). Separation mechanisms for proteins in RP-HPLC have also been proposed [1–10]. According to those studies, the behaviour of proteins on RP-HPLC columns is very different from that of small solutes. In RP-HPLC, the retention times of small molecules generally increase with increasing alkyl chain length of the chemically bonded phase on the chromatography support. In contrast, it is known that the retention of proteins is not seriously influenced by variations in alkyl chain length [5,11,12]. Consequently, it suggested that proteins interact only with the extreme top of the alkyl chains, and they scarcely penetrate into the chemically bonded phase under certain conditions.

Various types of restricted access packings [13,14] have previously been developed for analysis with direct serum injection. Yoshida and co-workers [15,16] first reported a protein (bovine serum albumin)-coated ODS column for that purpose. The external surface of the support was treated with a denatured protein, and the internal surface still retained the characteristics of the reversed-phase column for small molecules. Pinkerton's group [17–19] proposed internal-surface reversed phases (ISRP), which have been widely used in therapeutic drug monitoring.

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Their material had both an external hydrophilic phase and an internal hydrophobic phase. Gisch et al. [20] prepared shielded hydrophobic phases (SHP), which had an external hydrophilic network that created a polyoxyethylene or polyethylene glycol. Hydrophobic zones such as phenyl groups were embedded in the polymer. Desilets et al. [21] reported semipermeable surfaces (SPS). Alkyl (C_8 and C_{18}) bonded phases were coated with Tween and Brij as non-ionic surfactants. Haginaka and co-workers [22,23] developed mixed functional phases (MFP) which had a phenyl phase as the hydrophobic phase and a diol phase as the hydrophilic phase on both external and internal pores. Thus the hydrophilic phases sterically prevent larger protein molecules from interacting with the hydrophobic zones, while small analytes are not hindered from interacting with the hydrophobic zones and are retained. Any restricted access packing has two functions: one restricts the access of large molecules to the hydrophobic bonded phase, and the hydrophobic function retains small molecules.

The above previous studies prompted us to consider a different restricted access separation system. We imagined that if a chemical bond on a support had two different parts at its top and bottom, a hydrophilic function in the upper part and a hydrophobic function in the lower part, such a chemically bonded phase should be usable as a restricted access packing material. A commercially available "diol phase", a glyceryl-propyl-bonded phase, consists of a hydrophilic ethanediol structure and a hydrophobic methoxypropyl structure at the upper part and the lower part, respectively. Therefore, it appeared that an internal hydrophobic layer hidden by an external hydrophilic layer existed on the support surface of the diol phase. Such a formation of the diol phase, called a "binary-layered phase", seemed to be suitable for use as a restricted access packing (Fig. 1).

In this study, we evaluated a conventional diol silica packing material as a restricted access packing with direct injection for the determination of drugs in human serum, and found it to be useful for that purpose. As a result, we propose the following concept: stationary phase(s) such as the diol phase that have two different functions at a single chemical bond on the support should be called "binary-layered phase packing(s)", and separation(s) on the binary-layered phase packing should be named "binary-layered phase liquid chromatography".

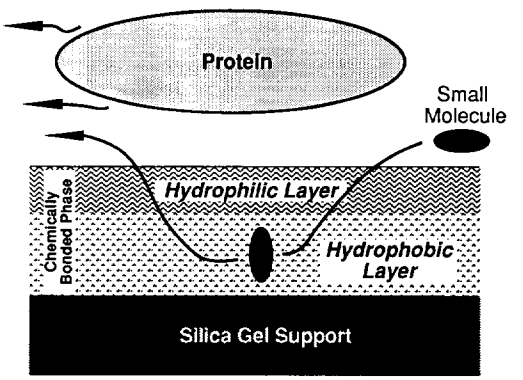

Fig. 1. Proposed conceptual model for binary-layered phase liquid chromatography using a binary-layered stationary phase as a restricted access packing.

2. Experimental

2.1. Reagents

Phenytoin sodium salt was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Other drugs and reagents were obtained from Wako (Osaka, Japan). HPLC-grade acetonitrile was from Kanto Chemical (Tokyo, Japan). Water was purified by passage through a Milli-R/Q system (Millipore, Bedford, MA, USA).

A diol silica packed cartridge column (LiChrospher 100 DIOL, 250 × 4 mm I.D., LiChroCART 250-4; Merck, Darmstadt, Germany) was obtained from Kanto Chemical. The mean particle diameter and pore size of this support were 5 μm and 10 nm, respectively.

2.2. HPLC apparatus and conditions

The HPLC system consisted of two Model 880-PU HPLC pumps (Jasco, Tokyo, Japan)

equipped with an ERC-3510 degasser (Erma, Tokyo, Japan), a Model 880-30 solvent mixing module (Jasco), a Model 7125 injector (Rheodyne, Cotati, CA, USA) and a Model 875-UV spectrophotometric detector (Jasco). An in-line filter unit, which had a changeable PTFE paper filter, was provided between the injector and the column to guard the column. Chromatograms were recorded and processed by a C-R6A Chromatopac integrator (Shimadzu, Kyoto, Japan).

The mobile phases, flow-rate and other chromatographic conditions used are given in the figure legends. All separations were performed at room temperature.

2.3. Sample preparation

Drugs were added to human serum at a known concentration and the resulting serum was filtered through a 0.22-μm membrane filter. An aliquot of the serum sample (a few to 20 μl) was then injected directly into the HPLC system.

3. Results and discussion

Many attempts have been made to apply direct serum injection in therapeutical drug monitoring using restricted access packings [13–23]. In these investigations, the procedure for sample pretreatment was extremely simple; the only filtration required used a membrane filter. The mobile phase composition in the separation systems was also uncomplicated. A mixture of a neutral buffer with an organic modifier such as acetonitrile was generally used as the mobile phase, and an acidic buffer or ion-pair technique was often used for the determination of ionic drugs. The content of the organic modifier was low, a few to 20%, because a high concentration of the organic solvent frequently caused precipitation of serum proteins. This study followed the previous examinations regarding the conditions for sample pretreatment and chromatographic separation.

Diol silica packings with macropores (mean pore diameter $\geq$30 nm) have been generally used for the size-exclusion separation of proteins [24]. In size-exclusion chromatography, it is a prerequisite that sample solutes have no direct interactions with the stationary phase on the support. Therefore, the proteins do not penetrate into the chemically bonded diol phase. Further, because the diol silica utilized here has micropores, the mean pore diameter being 10 nm, macromolecules such as proteins cannot permeate into the pores. Hence the proteins seem to be size-excluded and are eluted at the solvent front.

As a matter of course, extremely hydrophilic, water-attracting, small molecules such as amino acids and short-chain peptides were also separated according to the size-exclusion mode on the microporous diol phase eluted with an aqueous mobile phase without an organic modifier. However, relatively hydrophobic solutes behaved differently. In a preliminary examination, various amino acids and their homo-polypeptides were separated on the diol phase under aqueous elution conditions. Fig. 2 shows the relationship between the retention time of the solutes and the logarithm of their molecular mass. Glycine, alanine and their homo-polypeptides, which were relatively hydrophilic compounds, were separated based on the size-exclusion mode. However, the more hydrophobic leucine, phenylalanine and their polypeptides were eluted in reversed order. These retention data were replotted against Rekker's constant, which indicates the hydrophobicity of the compound (Fig. 3). For the phenylalanine derivatives, the retention time of the solute increased with increase in Rekker's constant. These observations suggest that the separation of hydrophobic solutes such as phenylalanine derivatives on the diol phase was based mainly on the reversed-phase mode. Further, simple series compounds, such as benzene, methylbenzene and ethylbenzene, were separated on the diol phase under various elution conditions. The correlation between the logarithm of the molecular mass of the benzene derivatives and their retention time is illustrated in Fig. 4. When an acetonitrile-rich (over 31.5%) mobile phase was used, the elution order of the compounds was based on the size-exclusion mode. It seems that, because the solutes were

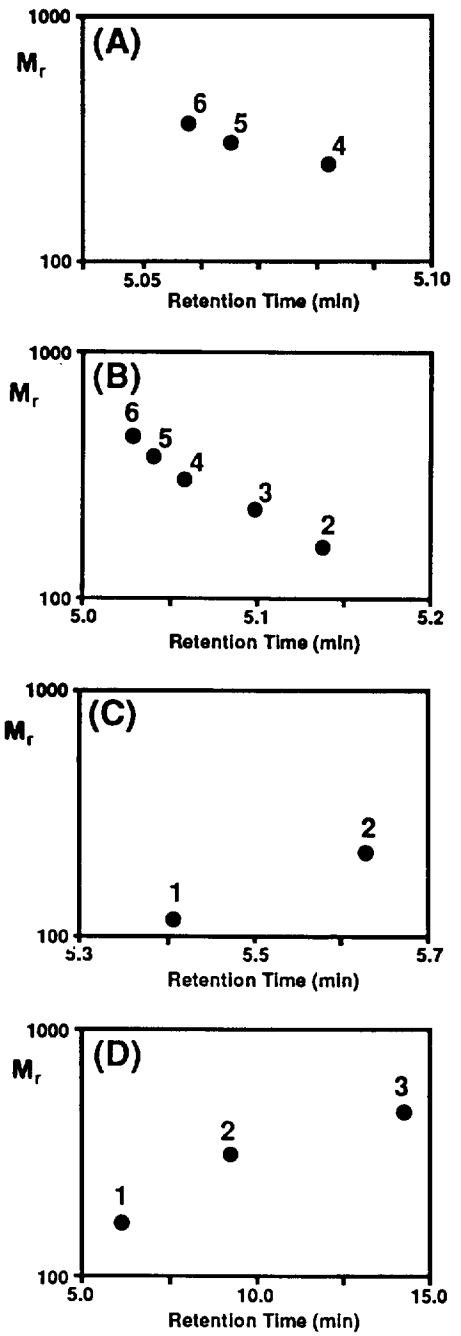

Fig. 2. Plot of molecular mass of the amino acid oligomers versus retention time on a diol silica gel column. Conditions: column, LiChrospher 100 DIOL (250 × 4 mm I.D.); eluent, 50 m*M* phosphate buffer (pH 6.9)–acetonitrile (91:9, v/v); flow-rate, 0.5 ml/min; column temperature, ambient; detection, UV at 215 nm. Samples, (A) $(Gly)_n$; (B) $(Ala)_n$; (C) $(Val)_n$; (D) $(Phe)_n$.

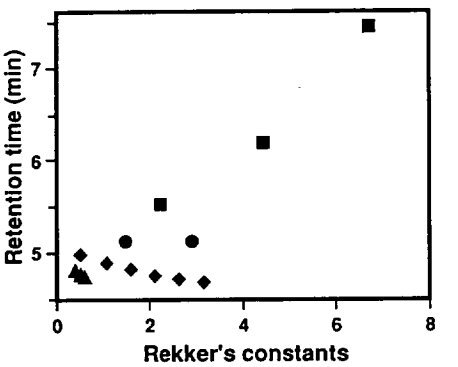

Fig. 3. Plot of retention time against Rekker's constants on diol silica gel column. Conditions as in Fig. 2. Samples, ▲ = $(Gly)_n$; ◆ = $(Ala)_n$; ● = $(Val)_n$; and ■ = $(Phe)_n$.

well solvated under these conditions, they had little interaction with the stationary phase. In contrast, the elution order was reversed by using an acetonitrile-poor mobile phase. Under these conditions, the solute compounds could penetrate into the hydrophobic layer of the chemically bonded diol phase and were retained based on the reversed-phase mode. These results suggest that such a diol-bonded phase behaves like two different types of packing materials considering

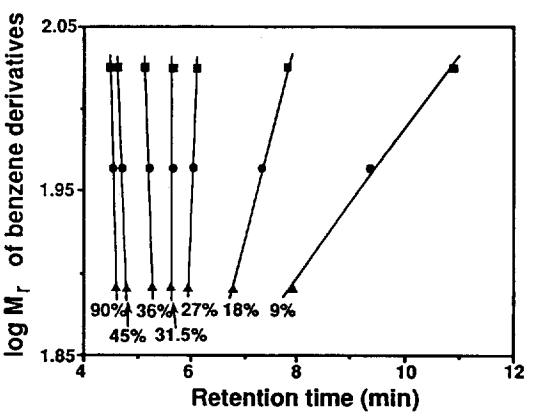

Fig. 4. Plot of logarithm of the molecular mass of the benzene derivatives against their retention times for different concentrations of acetonitrile in the mobile phase. Conditions: column, LiChrospher 100 DIOL (250 × 4 mm I.D.); eluent, 50 m*M* phosphate buffer (pH 6.9)–acetonitrile (percentages of acetonitrile are given below the lines); flow-rate, 0.5 ml/min; column temperature, ambient; detection, UV at 215 nm; injection volume, 2 μl. Samples: ▲ = benzene; ● = methylbenzene; ■ = ethylbenzene.

the state of the elution conditions or the physico-chemical characteristics of the solute compounds.

Next, standard human serum albumin and some small molecules such as synthetic drugs were injected into the diol column and were eluted with neutral phosphate buffer containing a small amount of acetonitrile. Chromatograms of the albumin and a mixture of theophylline and caffeine are shown in Fig. 5A and B, respectively. As expected, the albumin eluted at the solvent front, but the drugs were well retained and mutually separated from each other. The elution order of the drugs on the diol phase was almost the same as that on a conventional reversed-phase alkyl-bonded phase. Further, increasing the acetonitrile content in the mobile phase decreased the retention times of the drugs, and the drugs seemed to be separated mainly based on the reversed-phase mode. Strictly, the separation may be affected by some other physico-chemical interactions except for the hydrophobic interaction. As a result, it was found that the diol phase had two different layered functions to restrict the proteins and to retain the drugs. Therefore, the diol phase seemed to be suitable for a binary-layered phase packing as a restricted access packing material.

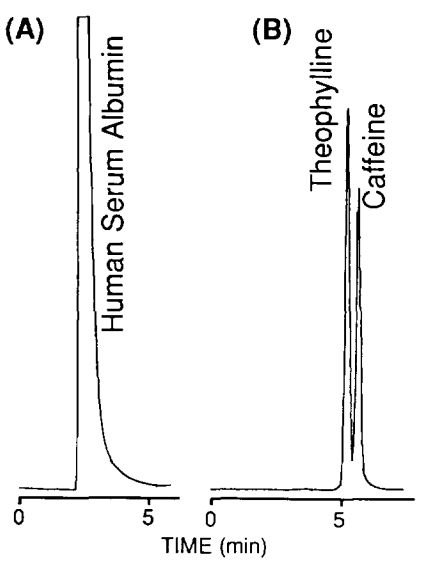

Fig. 5. Separations of (A) human serum albumin and (B) drugs on diol silica gel column. Conditions: column, LiChrospher 100 DIOL (250 × 4 mm I.D.); eluent, 50 m*M* phosphate buffer (pH 6.9)–acetonitrile (98.2:1.8, v/v); flow-rate, 0.6 ml/min; column temperature, ambient; detection, UV at 254 nm; injection volume, 20 μl. Samples: (A) human serum albumin (1%, w/v); (B) theophylline and caffeine (10 μg/ml each in water).

Fig. 6 shows chromatograms from the direct injection of human serum spiked with theophylline and caffeine on the diol silica column. Fig. 6A is for the serum bland and Fig. 6B for the spiked sample. The serum proteins were eluted in the void volume as the standard serum albumin, while the drugs followed the elution of the serum proteins, and these relatively hydrophilic compounds were well separated, as shown in Fig. 6B.

Fig. 7 shows a chromatogram from the direct injection of human serum containing phenobarbital, phenytoin and carbamazepine on the diol silica column. These relatively hydrophobic drugs were completely resolved within 10 min by increasing the acetonitrile content.

Fig. 8 shows the satisfactory separation of the acidic drugs acetylsalicylic and salicylic acid on the diol silica column with an acidic eluent.

When a macroporous diol silica gel (pore size ca. 30 nm) column was used for the same

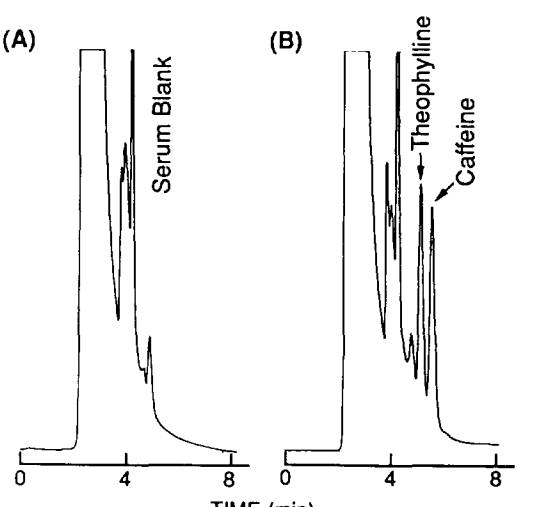

Fig. 6. Binary-layered phase liquid chromatographic analysis of (A) a serum blank and (B) serum spiked with drugs on a diol silica gel column. Chromatographic conditions as in Fig. 5. Samples: theophylline and caffeine (10 μg/ml each).

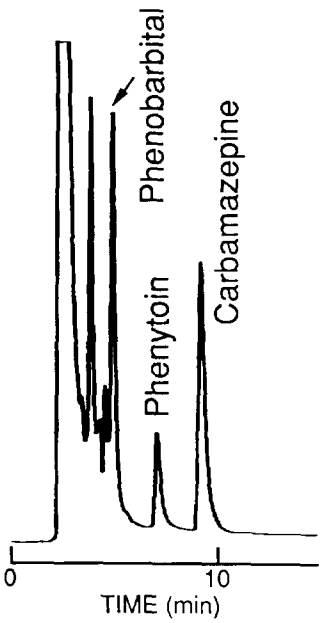

Fig. 7. Binary-layered phase liquid chromatographic separation of drugs in human serum by direct serum injection. Conditions: eluent, 50 m*M* phosphate buffer (pH 6.9)–acetonitrile (88:12, v/v); injection volume, 5 μl; other conditions as in Fig. 5. Samples: phenobarbital (18 μg/ml), phenytoin (20 μg/ml) and carbamazepine (18 μg/ml).

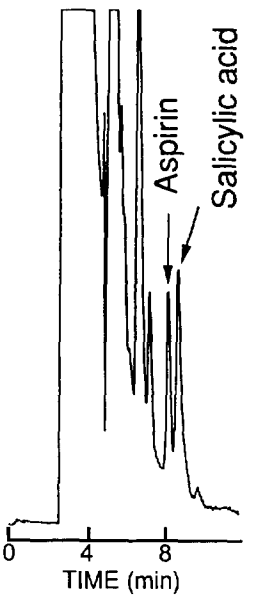

Fig. 8. Binary-layered phase liquid chromatographic separation of drugs in human serum by direct serum injection. Conditions: eluent, 50 m*M* phosphate buffer (pH 3.0)–acetonitrile (98.5/1.5, v/v); flow-rate, 0.5 ml/min; injection volume, 2 μl; other conditions as in Fig. 5. Samples: acetylsalicylic acid and salicylic acid (20 μg/ml each).

purpose, proteins also eluted before small molecules. However, small molecules such as drugs could not be sufficiently retained on the column to be mutually separated. The reason may be that the retention capacity of the macroporous diol silica is lower than that of microporous material based on the difference in their surface area. Although small hydrophobic solutes such as alkylbenzenes are naturally well retained on the macroporous diol silica column, it seens that these conditions are not practical for drug determinations.

As described above, diol columns have been used for the size-exclusion chromatography of the proteins. The recovery of the proteins on a diol column has also been investigated [25,26]. Schmidt et al. [25] reported that the recoveries of a number of proteins from a diol column were excellent when using 0.35 *M* sodium acetate (pH 5.0) and 0.1 *M* sodium sulfate as the mobile phase. Haginaka and Wakai [23] reported that serum proteins were almost completely eluted from a mixed functional phase containing a diol phase when 50–100 m*M* phosphate buffer over the pH range 3–7 was used as the eluent. The serum proteins seemed to be substantially recovered from the diol column used in the present study; the recovery of standard human serum albumin from the present diol column was nearly 100%.

When the PTFE paper filter of the column guard unit was changed after every 40th direct injection, the separation system with the same diol column maintained its performance for at least several hundred injections. Therefore, the binary-layered diol phase as a restricted access packing material seemed to be useful for direct serum injection for the determination of drugs.

As indicated above, it was found that the ethanediol layer if the diol-bonded phase restricted the access of proteins into the stationary

phase, and the methoxypropyl layer of the diol packing had sufficient hydrophobicity to retain small molecules such as synthetic drugs. As the hydrophobicity in the diol phase is lower than that for other previous restricted access packings, hydrophilic drugs such as theophilline eluted in the serum blank. However, this should not interfere with the quantitative analysis. Fig. 9 shows a suggested illustration of binary-layered phase liquid chromatographic separation on the diol-bonded silica column. In addition, diol-bonded silica gel was useful as a restricted access packing for the direct injection analysis of serum for the determination of both hydrophobic and hydrophilic drugs over the pH range of the eluent that is employed with ordinary siloxane-bonded silica material.

Fig. 10 shows schematic models of the restricted access packings. Previously reported models are based on the idea shown in model A, B or C. In model A, proteins were restricted by small pores from access to the internal hydrophobic bonded phase. This type of packing has two different functions on the internal surface and the external surface of the pores. Model B or C has a steric or shielding hydrophilic function that shielded the hydrophobic function to restrict protein access. These functions are introduced or localized without distinction regarding the internal or external location of the pores. In contrast, the present idea of a binary-layered phase is illustrated as model D. This is a new concept for a restricted access separation mechanism different from any idea of a multiple retention mechanism.

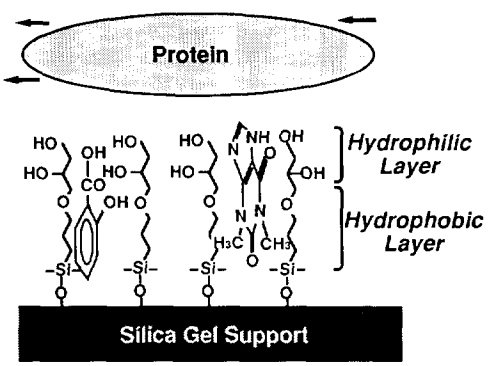

Fig. 9. Suggested illustration of the mechanism of the binary-layered phase liquid chromatographic separation of protein and synthetic drugs on the diol-bonded silica column.

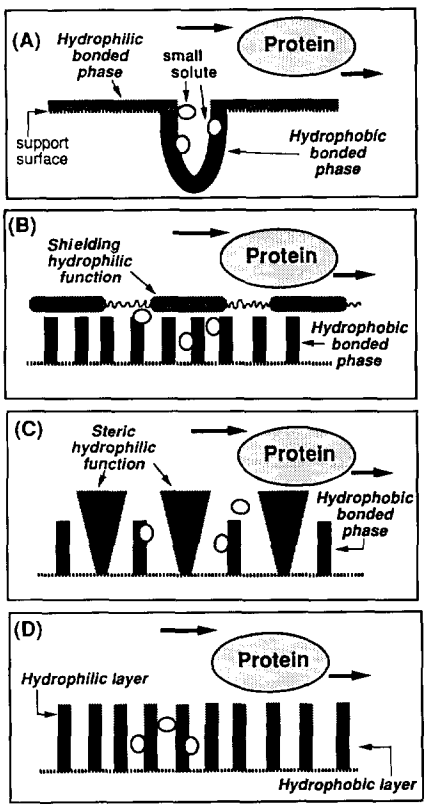

Fig. 10. Schematic models of various restricted access packings. (A) Internal-surface reversed phase; (b) shielded hydrophobic phase or semi-permeable surface; (c) mixed functional phase; (D) binary-layered phase.

Previously, many ideas have been reported for multiple retention mechanisms, and have been called "mixed-mode separation" [27], "multimode separation" [28], "bimodal separation" [29], and so on. These characteristic separations were carried out with various multi-functional stationary phases. For example, a long-chain alkylamine-coated alkylsilyl silica gel functioned as a hydrophobic anion exchanger, and the separations on this material were called mixed-mode separation. A polymer bead having a hydrophilic surface exhibited a negative hydrophobic effect owing to the constructional charac-

ter of the base gel, and the characteristic separations on the polymer gel were called multimode separation.

In contrast, the mechanism of the present multi-functional separation system can be clearly explained and discriminated from the above-mentioned previous multiple retention mechanisms. Two different functional structures were obtained in one chemical bond forming a binary-layered phase on the support surface, and these intended functions were displayed in one chromatographic process. Following the present idea, various other combinations of the different functions can be designed to prepare a variety of binary-layered phase packing materials. We therefore propose the following concept [30]: stationary phase(s) such as the diol phase that have two different functions at a single chemical bond on the support should be called "binary-layered phase packing(s)", and separation(s) on the binary-layered phase packing should be named "binary-layered phase liquid chromatography".

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