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# Possible Solution to Discord between Void Volume Recovery of Polypeptide and Separation of Drugs with Restricted-access Type Polymer-based Separation Media for HPLC

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Size monodisperse restricted-access and permeable polymer-based separation media were prepared and utilized as HPLC packing materials for void volume recovery of polypeptides and chromatographic separation of drugs. Restricted-access polymer-based packing material gave quantitative recovery of the polypeptide, but separation of some drugs was very poor, while permeable packing material with sufficiently large pores showed good recovery of the polypeptide and also excellent separation of the drugs.

Silica-based restricted-access reversed-phase packing materials have been recently developed and utilized in direct chromatographic analyses of drugs existing in serum or plasma.[1] In those packing materials, only small pores (80 Å or less) which can exclude large molecules such as polypeptide are involved, therefore only permeable small molecules can be separated in the pores mainly due to hydrophobic interactions. Although those packing materials usually involve hydrophobic internal surface and hydrophilic external surface, a mixed functional silica support where both hydrophobic and hydrophilic ligands coexist on internal and external surfaces can be also utilized to afford good results.[2] In addition, "binary-layered phase"[3a] and chemically stable polymer-based separation media[3b] involving homogeneously hydrophilic surfaces are recently utilized.

Generally, a disadvantage of polymer-based packing materials is that those with large contribution of micropores which are representatives of typical restricted-access packing materials tend to show peak broadening and size exclusion effect toward small molecules as well as large molecules.[4] From this point of view, restricted-access type polymer-based separation media are not always suitable for separation of drugs. Basically, with restricted-access type polymer-based packing materials, polypeptide can be recovered due to hydrophilic ligand (e.g. -OH group), while polymer back born which is composed of alkyl chain acts as hydrophobic ligand to achieve a separation of small molecules through a hydrophobic interaction. Therefore if polymer-based separation media have chemically homogeneous and hydrophilic external and internal surfaces, permeable packing materials are also expected to have preferable characteristics of restricted-access packing materials. Here, we wish to report chromatographic attempt for application of permeable polymer-based separation media as packing materials to direct analysis of drugs existing with polypeptide to improve potential dispdvantage of microporous particles.

Four polymer-based separation media with different pore size were prepared using commercial glycerol dimethacrylate as only monomer by a typical two step swelling and polymerization method.[5] Although utilized monomer was relatively hydrophilic, the polymerization in aqueous media afforded almost quantitative yields of

polymer beads with nice size monodispersity. Prepared four kinds of beads were packed into stainless steel columns (4.6 mm ID X 150 mm) by slurry method.

In reversed-phase mode, if hydrophobic selectivity[6] in terms of the increase in retention caused by one methylene group of solutes, $\alpha(CH_2)$ is compared, the prepared polymer-based separation media showed almost identical hydrophobic selectivity as summarized in Table 1.

Table 1. Chromatographic Properties of Prepared Packing Materials[a]

| Packing Material | $\alpha(CH_2)$ | | T/O[b] |
|---|---|---|---|
| | Ph-R[c] | R-OH[d] | |
| **1** | 1.31 | 1.34 | 1.50 |
| **2** | 1.31 | 1.32 | 1.42 |
| **3** | 1.32 | 1.33 | 1.66 |
| **4** | 1.32 | 1.30 | 1.57 |

a) Mobile phase, 40% aqueous acetonitrile; Flow rate, 0.8 ml/min.

b) k'(triphenylene)/k'(o-terphenyl); c) k'(amylbenzene)/k'(butylbenzene).

d) k'(decylalcohol)/k'(nonylalcohol).

Moreover, stericselectivity in terms of $\alpha$ value between planer triphenylene and sterically bulky but similarly hydrophobic o-terphenyl (T/O) was found to be similar.[6] These findings indicate the prepared packing materials have chromatographically identical surfaces which mainly determine the retention selectivity to small molecules.

Pore size distributions of the prepared particles determined using inverse size exclusion chromatographic method[7] were schematically depicted in Fig. 1. Although pore size distribution of packing material **3** was not included in Fig. 1 to avoid a confusion, pore size was well controlled and packing material **1** was found to be restricted-access type packing material, while packing material **4** was thought to be permeable packing material where exclusion limit was around MW=500000. Packing material **2** happened to have borderline pore size where average pore size was 85 Å in diameter, while packing material **3** had 109 Å as average pore diameter which was the middle pore size between packing materials of **2** and **4**.

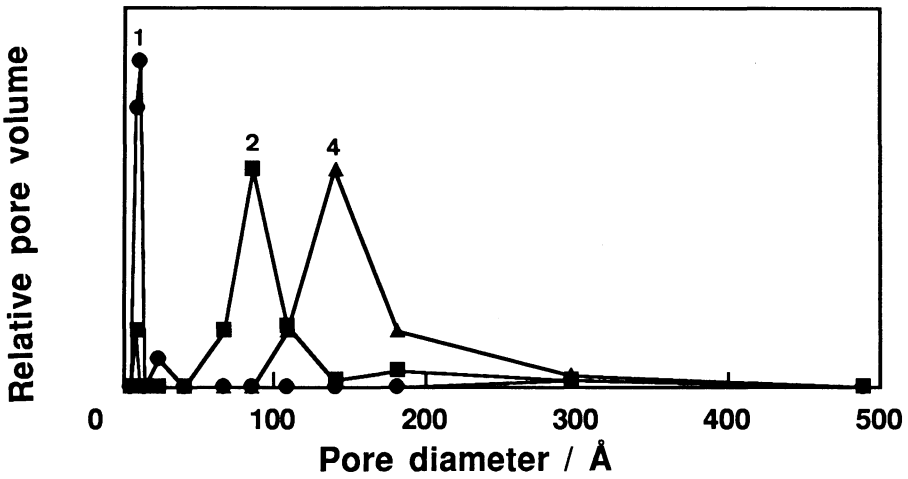


Fig. 1. Pore size distribution of the prepared particles.

A typical example which demonstrates disadvantage of microporous polymer-based packing material **1** can be found in separation between barbital and phenylbutazone. As shown in Fig. 2, both drugs could be well separated with good peak shape on the macroporous packing material **4** (a), while with the microporous packing material **1** (b), both drugs could not be separated and the peak shape was also very broad as predicted before. This is because micropores involved in **1** exclude relatively bulky molecule, phenylbutazone (2) which leads peak overlapping with barbital (1). Moreover, due to slow diffusion in the micropores causes peak broadening.[4)]

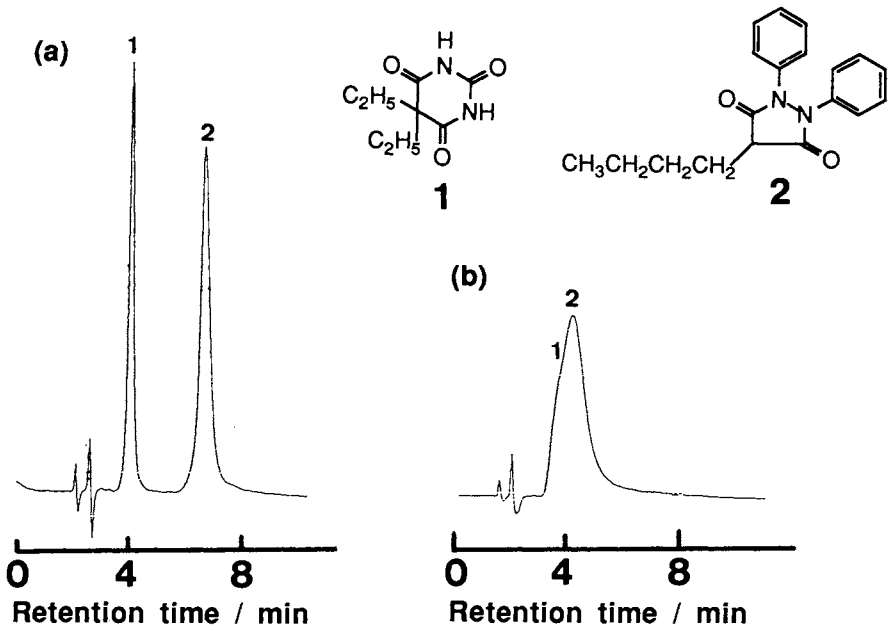


Fig. 2. Separation of drugs. (a), with **4**; (b), with **1**
Chromatographic conditions; Mobile phase, 30% aqueous acetonitrile-0.02 M phosphate buffer containing 0.1 M $Na_2SO_4$; Flow rate, 0.8 ml/min.; Detection, UV 254 nm; Samples, 1, barbital; 2, phenylbutazone.

Recovery of two polypeptides was measured by two methods of peak area of the eluted polypeptide at void volume and Coomassie Brilliant Blue G-250 (CBB) method.[8)] These data were summarized in Table 2.

Table 2. Recovery of Polypeptide with Prepared Packing Materials[a)]

| Packing Material (Pore diameter / Å)[b)] | Recovery / % | | | |
|---|---|---|---|---|
| | BSA[c)] | | Cytochrome C | |
| | Peak area[d)] | CBB[e)] | Peak area[d)] | CBB[e)] |
| **1** (10) | 103 | 99 | 106 | 103 |
| **2** (85) | 24 | 73 | 61 | 93 |
| **3** (109) | 44 | -----[f)] | 105 | 98 |
| **4** (160) | 59 | 85 | 103 | 101 |

a) Mobile phase, 10% aqueous acetonitrile-0.02 M Phosphate buffer containing 0.1 M $Na_2SO_4$, pH=7; Flow rate, 0.8 ml/min.; Injected polypeptide, 100 µl solution containing 20 mg/ml of the polypeptide. b) Average pore diameter determined by inverse size exclusion method.
c) Bovine serum albumin. d) UV absorption at 280 nm.
e) Fraction from 1 to 7 min after injection was collected to the test. f) Not determined

Although hydrophobic selectivity on all packing materials was identical, recovery of polypeptide was affected by the pore size of the packing materials. Since complete recovery on packing material **1** was observed, surface of the packing materials was clearly enough hydrophilic to elute polypeptide. Interestingly, recovery of BSA on packing material **2** was the lowest by peak area analysis and as average pore diameter became larger, better recovery was obtained. Since CBB method gave much higher recovery on **2**, this low recovery by peak area analysis is due to slow diffusion of polypeptide[9] in the pores and delayed elution of polypeptide which might prevent fine analysis of retained small molecules is happened. Interestingly, smaller polypeptide, cytochrome C was also badly recovered with **2**, while **4** gave quantitative recovery. These findings strongly predict that packing materials involving wider pores than **4** can give quantitative recovery even for BSA and those packing materials can enhance loading capacity in comparison with restricted-access type packing materials where eluted polypeptide may cause severe peak tailing which affects resolution of hydrophilic solutes.

Here, the amount of injected polypeptide was not large enough for practical analyses, however, if the packing material has sufficiently large pores, permeable packing materials like **4** are also found to have an ability for direct analysis of drugs in serum or plasma. Optimization in pore size as well as development of more suitable monomers is now under progress. Finally, the authors are grateful to Dr. Jun Haginaka of Mukogawa Women's University for helpful comments and useful suggestions.

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