効果の違いを検討することにより、今後の研究の参考になることを期待しております。

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Effect of Water Content in Mobile Phase on Chromatographic Behaviors of Low Molecular Compounds in RP-HPLC using Methacrylate-based Monolithic Column

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Abstract
The effect of water content in a mobile phase on chromatographic behaviors was studied with a monolithic stationary phase of poly(butyl methacrylate-co-ethylene dimethacrylate). A drastic decrease in the separation efficiency was observed when the water content was up to 90%, i.e., 10% acetonitrile (ACN) aqueous solution. Interestingly, the magnitude of the efficiency decrease significantly depended on the retention factor of the analytes, in particular, the separation efficiencies of the slightly retained compounds (0.1 < k < 1, in 10% ACN) were inferior. Furthermore, with a mobile phase of 10% ACN, the acceleration of the flow rate decreased the retention factor of these less retained compounds. The higher flow rate suppressed the interaction in RP-HPLC only when the water rich solution was used. The variation in the retention factor produced non-theoretical relations in an H-u plot. The change in the monolith structure was also indicated by the variation in the permeability in 10% ACN.

Keywords: Methacrylate monolith; Water content; Separation efficiency; Retention factor; Permeability; Flow rate

1. Introduction
Today, monolith columns have attracted and numerous papers have been published in a field of HPLC [1-4]. Monolithic columns can be divided into two types, i.e., silica and polymer ones. In general, separation efficiency of a silica monolithic column is superior to that of polymer one. However, recent progresses of polymer monoliths achieved their separation efficiency compatible with a silica monolith. Hosoya et al. prepared epoxy-based polymer monolithic column with high separation efficiency of 130,000 plates/m [5]. In our laboratory, we prepared methacrylate-based monolithic columns by low temperature UV photo-polymerization [7-9], of which column efficiencies were reached up to 105,000-160,000 plates/m with t0 marker and alkylbenzenes [9].

Other possible disadvantage of polymer monolithic columns over silica ones is a swell/deswell property. That is, the polymer monolith often changes its structure depending on a composition of a mobile phase. It is known that the variation in monolithic structure affects both flow resistance (permeability) and separation efficiency. To suppress the swell/deswell of a polymer monolith, Lubbad and Buchmeiser prepared the monolith using only a monomer with tetra-reactive functional groups and successfully depressed the change in swelling propensity [10]. However, general polymer monoliths are prepared with a mixture of monomers to provide retention and cross-linking abilities, and this type of a polymer monolith is difficult to suppress the problems based on a swell/deswell behavior. As an instance, in our laboratory, the drastic decrease in separation efficiency of caffeine and other compounds were observed when a mobile phase of 90% water content was used whereas the monolithic column had achieved good separation efficiency over 60,000 plate/m for alkylbenzenes with 50% ACN aqueous mobile phase, as described later.

In this study, the effect of water content in a mobile phase on chromatographic behavior was investigated with...
low molecular compounds as prove analytes. As a composition of a monolith, a combination of butyl methacrylate (BMA) and ethylene dimethacrylate (EDMA) was used because poly(BMA-co-EDMA) is one of the most general composition as a polymer-based monolithic column.

2. Experimental

2.1. Chemicals

Butyl methacrylate (BMA), ethylene dimethacrylate (EDMA), 2,2-dimethoxyphenyl-2-acetophenone (DMPA), 3-methacryloxypropyltrimethoxysilane (MAPS, Shin-Etsu Chemicals, Tokyo, Japan), 1-decanol, cyclohexanol, and methanol were used for the column preparation. As analytes, uracil ($t_0$), toluene (TE), $n$-propylbenzene (PrB), $n$-pentylbenzene (PnB), caffeine (CF), 1-methylxanthine (MX), theophylline (TP), phenol (PN), resorcinol (RC), $o$-cresol (CS), benzoic acid (BA), salicylic acid (SA) were used. The mixture of acetonitrile (ACN) and distilled water was used as mobile phases. All reagents except for MAPS were purchased from Wako Pure Chemicals (Osaka, Japan). All chemicals were used as received.

2.2. Column preparation

The poly(BMA-co-EDMA) monolithic column was prepared by UV-photo initiated polymerization under low temperature. The preparation process was almost the same as our previous paper [7]. A UV-transparent fused silica capillary (i.d. 0.1 mm, o.d. 0.375 mm, GL Science, Tokyo, Japan) was silanized with MAPS. The capillary was filled with reaction solution consisting of BMA (monomer, 24 wt%), EDMA (cross-linker, 16 wt%), 1-decanol (porogen, 34 wt%), cyclohexanol (porogen, 26 wt%), and DMPA (photoinitiator, 1 wt% with respect to the monomers). The monolith was prepared by photopolymerization using a UV illuminator (3UV Benchtop Trance Illuminator, Upland, CA, USA, 254 nm) for 8 min in an incubator (MIR-153, Sanyo, Osaka, Japan) at -15°C. After polymerization, the columns were immediately washed with methanol to remove unreacted reagents and porogens. The monolithic part of the capillary was cut to 10 cm long before the use as a HPLC column.

2.3. Chromatograph

A capillary HPLC used in this study consisted of a pump (LC-20AD, Shimadzu, Kyoto, Japan), a sample injector (Model 7520, Rheodyne, Cotati, CA, USA) with a 0.5 μL sample loop, a splitter for split injection (resistance capillary: 30 cm x i.d. 0.05 mm), and a UV detector (CE-2075, Jasco, Tokyo, Japan, 210 nm).

3. Results and Discussion

3.1. Typical effect of water content on separation efficiency

A typical effect of water content in a mobile phase on the separation efficiency was shown in Fig. 1. A separation of $t_0$ marker (uracil) and resorcinol (RC) with the mobile phase of 50% ACN aqueous solution was shown in Fig. 1A. In this mobile phase, RC was eluted as a sharp peak ($N = 60,000$ plates/m, $k = 0.1$). When the ACN content in the mobile phase decreased down to 10%, the column efficiency for RC drastically decreased ($N = 2,400$ plates/m, $k = 1.0$) as shown in Fig. 1B. Since it is well-known that column efficiency depends on a retention factor of an analyte (in this case, the retention factor of RC increased from 0.1 to 1.0 in the water rich mobile phase), here, the column efficiency for theophylline (TP) was also evaluated as a less retained analyte ($k = 0.05$) in the mobile phase of 10% ACN aqueous solution (Fig. 1C). Although the retention was low enough, the column efficiency for TP was low (6,200 plates/m) as similar to RC in Fig. 1B. This phenomenon indicated that the separation efficiency with the poly(BMA-co-EDMA) monolith depressed when a water content of a mobile phase increased.

As the further study of effect of water content on the separation efficiency, the relationships between log $k$ and log $H$ (theoretical plate height in μm unit) of various compounds were studied with mobile phases of 50% and 10% ACN aqueous solutions. As clearly shown in Fig. 2, the separation efficiencies with the mobile phase of 10% ACN (triangle) was significantly inferior to those with the mobile phase of 50% ACN (circle) over whole log $k$ region (from 0.016 to 80 in $k$ value). For example, when ACN content changed from 50 to 10%, the $H$ values of CA drastically increased from 17 to 1000 μm (58 times) together with the slight increase in $k$ (from 0.03 to 0.14).
On the other hand, although the $k$ value for TE was increased from 1.4 to 80 in 10% ACN mobile phase, the variation in $H$ value for TE was relatively small, i.e., the $H$ values in 50% and 10% ACN were 16 and 45 $\mu$m (2.8 times), respectively. The column efficiency of $t_0$ marker also decreased with the increase in water content of a mobile phase, but the variation in the $H$ values of $t_0$ was small, i.e., $H = 13$ and 23 $\mu$m (1.8 times) for 50% and 10% ACN mobile phases, respectively.

With 50% ACN mobile phase (circle), the separation efficiency ($\log H$) less depended on the kind of analytes with various retention factors ($k = 0.03$ to 7), i.e., $H$ values for all retained analytes were ranged from 14 to 19 $\mu$m. In contrast, in the case of 10% ACN mobile phase, the $H$ values significantly depended on the kind of analytes, i.e., the $H$ values in 10% ACN mobile phase ranged from 22 to 1000 $\mu$m. The behavior of separation efficiency in 10% ACN mobile phase was different from that in 50% ACN mobile phase. As is interesting to note, the relation between $\log k$ and $\log H$ produced the maximum at $\log k = -0.85$ ($k = 0.14$, CF) in 10% ACN solution. The separation efficiencies of slightly retained analytes ($0.1 < k < 1$, or $-1 < \log k < 0$) were significantly low in a mobile phase of 10% ACN.

Here, for the further investigation, we selected uracil ($t_0$), caffeine (CF), resorcinol (RC), and toluene (TE) as the prove analytes. CF and RC were slightly retained compounds of which $H$ values were significantly inferior in 10% ACN mobile phase and TE is an analyte with relatively superior $H$ and the higher retention.

### 3.2. Effect of water content on $H-u$ curve

The separation efficiencies of the analytes decreased with a poly(BMA-co-EDMA) monolithic column using a mobile phase of 10% ACN as described in the previous section. For the further investigation, relationships between flow velocity ($u$) and theoretical plate height, so-called van Deemter plot, was studied. The $H-u$ plots of four prove analytes ($t_0$ circle), CF (diamond), RC (triangle), and TE...
(rectangle)) were shown in Fig. 3 with three mobile phases containing (A) 50%, (B) 20%, and (C) 10% ACN. In Fig. 3A and B, the relationships of all analytes can be found as a general model based on an equation of \( H = A + B/u + Cu \). In the mobile phase of 50% ACN (Fig. 3A), the retention factors of analytes were significantly low (\( k_{CF} = 0.03, k_{RC} = 0.07 \)). Therefore, it will be reasonable that the \( H-u \) plots of CF and RC almost agreed with that of \( t_o \). Interestingly, the \( H-u \) plot of TE (\( k_{TE} = 1.4 \)) was also compatible for other three “non-retained” compounds in Fig. 3A (50% ACN). This result would suggest the fast mass transfer of TE between mobile and stationary phases in this condition.

When the ACN content was down to 20% (Fig. 3B), the separation efficiency decreased and the dependence of the \( H-u \) plot on a kind of analytes were clearly observed in particular for C terms. The C terms for CF (diamond) and RC (triangle) were larger than that for TE whereas their retentions to the stationary phase were smaller than that for TE.

As described above, all \( H-u \) curves shown in Fig. 3A and B were based on the general relationship of \( H = A + B/u + Cu \). However, the plots for CF and RC were drastically changed when the ACN content decreased to 10% (or water content increased up to 90%) as shown in Fig. 3C. In Fig. 3C, the relationship for \( t_o \) and TE were general curves based on \( H = A + B/u + Cu \). Moreover, the C term in the \( H-u \) curve for \( t_o \) in 10% ACN mobile phase (Fig. 3C) was almost compatible with that in 50% ACN (Fig. 3A). This behavior suggested that the structural change of the monolith, produced by the variation in a mobile phase composition, would less effect on the chromatographic behavior of a non-retained analyte. In contrast, the relationships for CF and RC were clearly unusual against to the basic theory. In the case of RC (triangle), the profile formed a convex curve reached to \( H = 5600 \) \( \mu \)m. Further unusual behavior was observed for CA in Fig. 3C. The \( H \) of CA increased convexly until \( u = 1 \) mm/s; then decreased till \( u = 4.7 \) mm/s. Over 5 mm/s, the \( H \) value slightly increased together with the increase in \( u \) as similar to \( t_o \) and TE. The chromatographic behavior of CF and RC were clearly against the basic chromatographic theory.

Here, the chromatograms of RC in 10% ACN at flow velocities of 1.0 and 10 mm/s were shown in Fig. 4. As is interesting to note, the retention of RC clearly decreased at the higher flow velocity, \( i.e., \) the \( k \) values for 1.0 and 10 mm/s were 0.95 and 0.63, respectively. The retention factor of CA also decreased from 0.13 to 0.06 by the increase in the flow rate. The dependence of a retention factor on a flow velocity was observed. This phenomenon was further investigated in the next section.

3.3. Effect of water content on \( k-u \) relation

The relationships between retention factor and flow velocity, \( \log k-u \) plots, of CF, RC, and TE were shown in Fig. 5. In the case of TE, the dependency of \( \log k \) on \( u \) was less observed, or almost constant, with all mobile phases of different ACN content. Needless to say, this behavior is general in HPLC. On the other hand, in the case of RC, the decrease in \( \log k \) was clearly observed only when the mobile phase containing 10% ACN (or 90% water) was used. Focused on CF, the decrease in \( \log k \) in 10% ACN mobile phase was also found but the variation pattern was not the same as that for RC. That is, \( \log k \) decreased rapidly until the flow rate of about 2 mm/s, then the slope became about 1/12 of the rapid one (see Table 1 for detail). The \( \log k-u \) plot of CF over 2 mm/s with 10% ACN was almost compatible with that with 20% ACN. Further increase in the ACN content reduced retentions of CF (\( k < 0.06 \)) and relatively large errors were found with both 30% and 50% ACN mobile phase.

Since the specific retention behavior was observed only with the mobile phase of 10% ACN, the \( \log k-u \) plots for 10% ACN was further investigated. The slope of decrease (\( d\log k/du \)), the estimated value of \( k \) at \( u = 0 \) (\( \log k_{00} \) and \( k_{uo} \)), and correlation coefficient were calculated and summarized in Table 1. Since the variation behavior of \( \log k_{CF} \) can be divided into two region, two sets of the values were listed in Table 1 (the flow rate ranges used for the calculations were also described). The slope value (\( d\log k/du \)) for CF (the slower region of 0.05-2 mm/s) was about 10 and 60 times of those for RC and TE, respectively (note that, for TE, the slope was almost zero and the less correlation was observed). Focused on CF and RC, the slope value of CF in the slower \( u \) range (0.05-2 mm/s) was 10 times longer than that of RC, but the slope of CF in the faster flow rate over 2 mm/s was the same as that of RC. The mechanisms of the retention reduction for CF would be
divided into two types and the mechanism at the higher region might be common with that for RC.

It is well-known that the logarithmic value $ln k$ is proportional to organic modifier content of a mobile phase in reversed phase HPLC (RP-HPLC). The relationships between ACN content and $ln k$ values of CF were shown in Fig. 6. At a flow rate of 1.0 mm/s (open triangle), the relationship was almost in linear and agreed with the theory. The same relationship was also observed with the lower flow rate of 0.5 mm/s (data not shown). However, with the flow rate of 2.0 mm/s (open diamond), the $ln k$ at 10% ACN were plotted clearly the lower position of the linear relationship observed in 20 to 50% ACN. The same behaviors were also observed for the higher flow rate of 5 and 10 mm/s (data not shown). This fact indicated that the retention of CF in 10% ACN mobile phase with the fast flow velocity was against to the general behavior in RP-HPLC. In Fig. 6, the estimated value of $ln k$ at the zero flow rate ($ln k_{uo}$), were also plotted. Since the log $k-u$ relation for 10% ACN was divided into the slow and fast flow rate regions as shown in Fig. 5, the estimated values from both the slower (open) and the faster (closed) regions were plotted for 10% ACN. In the fast region, the $ln k_{uo}$ for the faster region (marked with close circle) was plotted the lower position than the straight line. The hydrophobic interaction in 10% ACN would be inhibited when the flow rate increased over 2 mm/s.

| Parameters of decrease in retention shown in Fig. 5 with 10% ACN mobile phase. |
|------------------------|----------|--------|--------|-------------|
|                        | log $k_{uo}$  | $dlogk/du$ | $r^2$  | $u$ range [mm s$^{-1}$] |
| CF                     | -0.62 (0.24) | -0.23   | 0.997  | 0.05 - 2    |
| -1.04 (0.09)         | -0.02     | 0.975   | 2 - 15 |
| RC                     | -0.02 (0.96) | -0.02   | 0.997  | 0.05 - 13   |
| TE                     | 1.90 (79.8) | -0.00   | 0.660  | 2 - 12      |

Fig. 5. Relationships between flow rate ($u$) and retention factor ($log k$) of (A) caffeine, (B) resorcinol, and (C) toluene using mobile phases of 10% (open circle), 20% (closed triangle), 30% (open rectangle), and 50% (closed diamond) ACN aqueous solution. Other chromatographic conditions were the same as Fig. 1.

Fig. 6. Relationships between ACN content and $ln k$ of CF measured with the flow velocities of 1.0 mm/s (open triangle), 2.0 mm/s (open diamond), and 0 mm/s (open circle, estimated value from the $k-u$ relationship). As the estimated value at 10% ACN, both the slower (open circle) and faster (closed circle) regions were plotted. Other chromatographic conditions were the same as Fig. 1.
The variation in the retention factor, shown in Fig. 5, will be helpful to understand the unexpected behaviors of CF and RC in their H-u plots shown in Fig. 3C. In the case of RC, the retention factor decreased with increase in the flow rate as shown in Fig. 5B. However, the decrease in k from 1 (log k from 0) produced the inferior separation efficiency as shown in Fig. 2. Therefore, the significant increase in H would be observed in Fig. 3C according to the increase in the flow velocity. In the case of CF, the similar behavior was observed with the flow rate less than 0.15 mm/s. The further decrease in retention factor (k < 0.1, or log k < -1) provides the enhancement in separation efficiency as shown in Fig. 2. The lower retention of CF permitted the H-u behavior similar to t0 merker in the flow rate over 2 mm/s. In conclusion, unexpected behavior of CF and RC in the H-u plots was the results of the specific variation in their retention factors, or dependence of k on u.

3.4. Effect of water content on monolithic structure

As described in the previous section, the dependency of k on u was found. Since the variation in k may be produced by the pressure-induced decrease in a volume of stationary phase (or deformation), the relationship between flow rate and back pressure, or p-u plot, was investigated. However, the linear relations (r>0.999) was observed with all mobile phase of 10%, 20%, 30% and 50% ACN in the flow rate of < 15 mm/s. This result indicated that the monolithic structural changes in a through-pore level, or macro scale, were not induced by the increase in the flow velocity with all mobile phases.

The relationship between water content in a mobile phase and permeability (K = u\eta L/P, where \eta, L, and P were viscosity, column length, and pressure drop, respectively) was also measured. As shown in Fig. 7, the permeability increased together with the increase in the water content. This result indicated that the poly(BMA-co-EDMA) monolith shrunk in the mobile phase of the higher water content and this phenomenon would be general for polymer-based stationary phases. In interesting, the permeability elevated rapidly when the water content increased from 80 to 90%. That is, the structure of the monolith was drastically changed in this region. This variation would be related with the specific behaviors shown in Fig. 2 and Fig. 5. However, further study is necessary to reveal the relation between the structural variation and the behaviors of both retention and separation efficiency of the analytes.

4. Conclusions

In this study, the effect of water content in a mobile phase on chromatographic behaviors was studied with the monolithic stationary phase of poly(BMA-co-EDMA), which is one of the most common composition of the polymer monolithic column. When the water content was up to 90%, the separation efficiency was drastically decreased and its dependency on the retention factor was clearly observed. In particular, the efficiencies of less retained analytes, such as CF and RC, were low in 10% ACN. As is interesting to note, the retention factor of these less-retained compounds was function of the flow rate only when 10% ACN was used as a mobile phase. Further investigation focused on CF revealed that the interaction in RP-HPLC mode was suppressed when the flow velocity increased in 10% ACN. The measurement of permeability suggested the relatively large structural change of the monolith in 10% ACN. However, the change was less affected on the separation efficiency of t0 and relatively highly-retained analytes of TE. Although we guess that the structural change in the monolith would have an important role, further investigation is necessary to reveal the effect of water content in a mobile phase on chromatographic behaviors.

References
