T R O U B L E S H O O T I N G

Separation Artifacts III: Secondary-Retention Effects in Reversed-Phase Chromatography

JOHN W. DOLAN

This is the third in a series (1,2) of articles concerning separation artifacts (3). This month, the effects of silanol on retention and ways to minimize silanol-interaction problems will be considered. In addition, some retention problems resulting from trace-metals contamination and ion-exchange interactions in reversed-phase separations will be mentioned.

In a well-designed HPLC separation, sample compounds are retained in a single retention process. In reversed-phase chromatography, for example, the solute interacts hydrophobically with the nonpolar alkyl chains of the column packing. For silica-based packings, however, interaction of some sample compounds with silanol groups is also possible. This *secondary-retention process* (silanol interaction) can lead to band tailing. Two reasons for this kind of band tailing exist:

- Secondary retention often affects a limited number of retention sites, and these sites are quickly filled. More specifically, the column becomes quickly overloaded for compounds that interact strongly with secondary-retention sites.
- In some cases, the interaction of sample compounds with secondary sites is quite strong, and the sorption–desorption kinetics are slow. On the chromatogram, this effect can be seen as severely tailing bands.

Band tailing that results from secondary retention is probably the most common and serious example of misshapen peaks and is also an indication that sample retention (and separation) is likely to vary from column to column. Secondary retention is a major contributor to poor column-to-column reproducibility. For these reasons, secondary retention will be carefully examined, and its effects on different HPLC methods will be considered. Secondary retention in reversed-phase separations has been variously attributed to different types of silanol groups and to the presence of trace-metal impurities in the column packing. Silanol interactions will be discussed first.

SILANOL INTERACTIONS

Several good examples of silanol effects are given in Figure 1. In each case, standard mixtures of acidic (vanillylmandelic acid, homovanillylmandelic acid, salicylic acid), basic (procainamide, *n*-acetylprocainamide), or neutral (caffeine) compounds were injected. Two 15 cm × 4.6 mm reversed-phase columns were used (Supelcosil LC-18 and Supelcosil LC-18-DB; Supelco, Bellefonte, Pennsylvania). The DB column was specially processed to minimize retention (and tailing) of basic compounds.

Figures 1a and 1b show the separation on each column using a mobile phase that is 7% (v) acetonitrile–water (pH 3.5, phosphate buffer). The basic compounds procainamide (PA) and *n*-acetylprocainamide (NAPA) are strongly retained and tail so badly that they run into the baseline on the LC-18 column (Figure 1a); such retention characteristics are typical of basic compounds on most reversed-phase HPLC packings. On the LC-18-DB column (Figure 1b), these two basic compounds elute much earlier with improved — but still inadequate — peak shapes. It is believed that the strong retention and tailing of amines such as PA and NAPA in most reversed-phase systems results from two kinds of silanol interaction:

hydrogen bonding

$$R_3N: + HO\text{–}Si\text{–} \rightleftharpoons R_3N: \cdots H \cdots O\text{–}Si\text{–} \quad [1]$$

ion exchange

$$R_3NH^+ + (Na^+)^-O\text{–}Si\text{–} \rightleftharpoons (R_3NH^+)^-O\text{–}SI\text{–} + Na^+ \quad [2]$$

The increased retention and tailing of the basic compounds PA and NAPA in Figure 1a suggests that the silanols of the LC-18 column are more acidic than those of the LC-18-DB column (Figure 1b).

When secondary-retention effects such as those illustrated in Figure 1 are present, generally the addition of some mobile phase modifier that will preferentially interact with and block these secondary-retention sites (silanol groups in the present example) will resolve the problem most effectively. Amine additives such as triethylamine (TEA) are commonly used for this purpose, with small concentrations of the amine (1–20 m*M*) usually being effective. The results of adding 10 m*M* TEA to the mobile phase for the LC-18 and LC-18-DB columns are illustrated in Figures 1c and 1d, respectively; band tailing is significantly reduced, and bands for PA and NAPA are sharpened. These results are typical of many such reversed-phase separations of basic compounds. Presumably, the amine modifier (TEA) replaces the solute R_3N^+ in reactions 1 and 2, thereby effectively eliminating the silanol groups as sorption sites for sample compounds. Typically, 10–20 m*M* TEA is adequate for this purpose; higher concentrations produce little added effect.

The bands for acidic compounds homovanillylmandelic acid (HVA) and salicylic acid (SAL) (Figures 1a–1d) show considerable variation in width and peak asymmetry, and tailing of SAL is particularly pronounced on the LC-18-DB column. The tailing of SAL is not improved by adding TEA (compare Figures 1d and 1b), which presumably neutralizes the effect of acidic silanol groups, and suggests that different kinds of silanol groups are present on the silica surface, some of which preferentially bind bases — whereas others bind acids. One possibility for interaction is:

$$R\text{–}COO^- + HO\text{–}Si^*\text{–} \rightleftharpoons R\text{–}COO^- \cdots H \cdots O\text{–}Si^*\text{–} \quad [3]$$

in which ionized acids $R\text{–}COO^-$ undergo hydrogen bonding with a special kind of silanol group (–Si*–OH) or, alternatively,

$$R\text{–}COOH + {}^-O\text{–}Si^*\text{–} \rightleftharpoons R\text{–}COO^- \cdots H \cdots O\text{–}Si^*\text{–} \quad [4]$$

There is substantial evidence for the existence of different kinds of silanol groups on the silica surface (4). If the previous hypothesis is correct, then the addition to the mobile

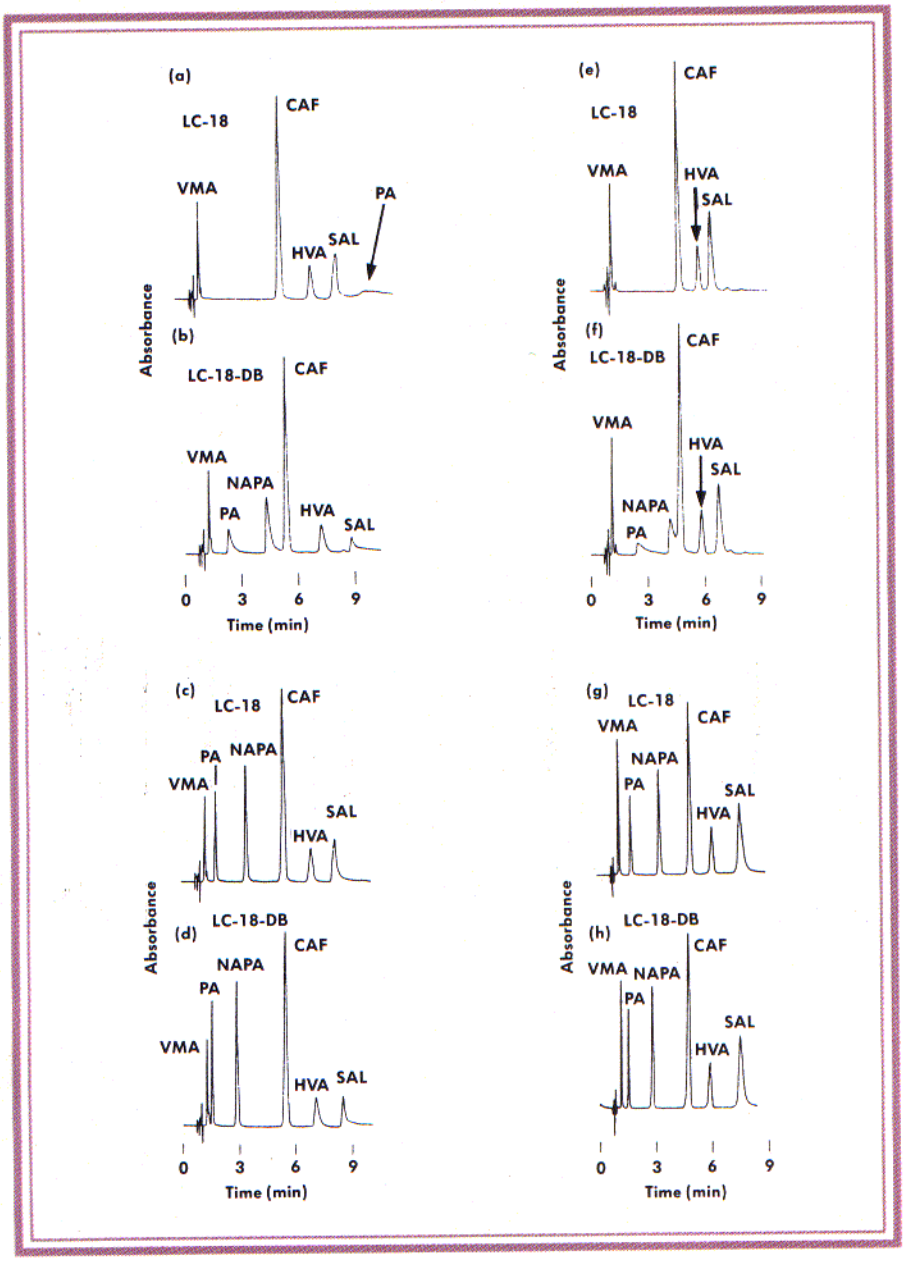

FIGURE 1: Secondary-retention effects in the reversed-phase separation of mixtures of neutral, acidic, and basic compounds. Columns: 15 cm × 4.6 mm Supelcosil LC-18 (Figures a,c,e, and g) and LC-18-DB (Figures b,d,f, and h); mobile phase: 7% (v) acetonitrile–water (pH 3.5), 10 m*M* TEA added in Figures c,d,g, and h and 1% acetate added in Figures e–h. Peaks: caffeine (CAF), neutral; homovanillylmandelic acid (HVA), vanillylmandelic acid (VMA), and salicylic acid (SAL), all acids; procainamide (PA) and *n*-acetylprocainamide (NAPA), both bases. (Courtesy of Supelco, Inc.)

phase of a carboxylic acid modifier should reduce the secondary-retention of acidic samples as in reactions 3 and 4. This possibility is tested in Figures 1e (LC-18) and 1f (LC-18-DB) by adding 1% acetic acid to the mobile phase, while holding pH constant. The acidic compounds HVA and SAL are now seen to produce quite sharp bands without discernible tailing. It appears that the addition of a carboxylic acid (for example, acetic acid) to the mobile phase improves band shape for carboxylic acid samples whenever the latter compounds show peak tailing.

Figures 1c–1f provide the following conclusions:

- Whenever band tailing that is caused by secondary retention is observed, addition to the mobile phase of modifiers having structures similar to the sample compound usually will reduce the effect. As the structure of the added modifier more closely approximates that of the sample compound, the effectiveness of the modifier in reducing tailing should increase correspondingly. This suggests that very severe cases of band tailing that are not resolved by addition of the usual modifiers may require special modifiers selected for their structural similarity to the sample compounds of interest.
- Samples that contain both acidic and basic compounds present a special problem. This case is seen in Figures 1e and 1f, in which addition of acetate to the mobile phase improves the band shape of the acidic components of the sample but worsens the shape of the basic compounds (increasing their retention). That is, acidic modifiers can intensify secondary-retention effects on basic sample compounds (cf. Figures 1b and 1f for PA and NAPA). In this case, the solution is to add both an acidic and a basic modifier to the mobile phase (Figures 1g and 1h; 1% acetate and 10 m*M* TEA). At this point, all bands in the chromatograms for either column (LC-18, Figure 1g; LC-18-DB, Figure 1h) are quite symmetrical. It should also be noted that retention for each compound in the sample is now almost identical for the two columns. In other words, the suppression of secondary-retention effects has also eliminated retention variability (compare Figures 1a and 1b).

Because basic compounds present band-tailing problems more often, there is particular interest in selecting the best amine modifier to minimize secondary retention. Recent systematic studies (5) have shown that secondary and, especially, tertiary amines with short alkyl groups (methyl or ethyl) are most effective in this regard. Although TEA has proved useful in a broad sense as an antitailing modifier for basic sample compounds, it sometimes fails to yield symmetrical bands, especially for certain columns and samples containing tertiary amines. For cases such as these, modifiers having the general structure $R(CH_3)_2N$ (where R is either hexyl or octyl) have proved to be more effective (6,7).

Although dimethylalkylamine modifiers such as dimethyloctylamine (DMOA) and dimethylhexylamine (DMHA) are often more effective in suppressing the secondary retention of amine samples, TEA is still a better first choice for several reasons. First, DMOA and DMHA are more strongly retained and may prove difficult to remove from the column when changing mobile phases for another assay procedure. This characteristic can cause retention variability for repeated injections of the same sample. Second, equilibrating the column with DMOA and DMHA can require more time than with more weakly held amine modifiers. Third, some workers have observed that the use of DMOA or DMHA improved the shape of tailing bands in a particular chromatogram but, at the same time, worsened the shapes of other bands. This behavior may not be typical, but it suggests that these modifiers may be likened to powerful drugs that produce serious side effects. To extend the analogy, DMOA and

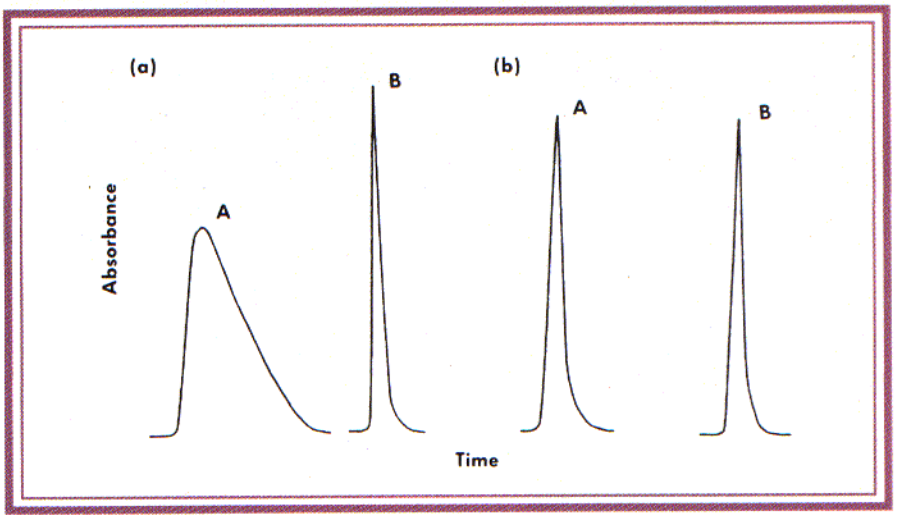

FIGURE 2: Heavy-metal contamination of the column packing. (a) Original column and (b) EDTA-washed column. Column: C18; mobile phase: 30% (v) acetonitrile–water, pH 6.7. Peaks: A = 2,3-dihydroxynaphthalene (a metal chelator) and B = 1,7-dihydroxynaphthalene (nonchelator). (Reprinted with permission from reference 11).

DMHA should not be used indiscriminately and should be considered only when the safer modifier TEA proves ineffective.

ION-EXCHANGE EFFECTS

Detailed studies by E. Papp and Gy. Vigh have shown conclusively that secondary retention can arise from the exchange of ions between protonated sample bases and ionized silanols (Equation 2) (8,9). Ion-exchange effects have been observed even for low-pH mobile phases (for example, pH 2.0), suggesting that some silanol groups are quite acidic. If it is suspected that secondary retention involving ion exchange is in effect, the problem can often be controlled by increasing buffer concentration in order to increase the ionic strength of the buffer, thereby driving the reaction of Equation 2 to the left.

TRACE METALS

A few studies suggest that some reversed-phase packings are contaminated with excessive amounts of heavy metals (iron, copper, and others) and that these metals give rise to secondary-retention effects that simulate the effects attributed to silanols (10,11). The elution of two dihydroxynaphthalene (DHN) derivatives from a particular C18 packing provides an example (Figure 2a). 2,3-DHN is capable of chelation with heavy metals and is seen to produce a band that is broader and displays more tailing than does the band for 1,7-DHN, which is incapable of chelation. After washing the column with the metal-chelator EDTA, the separation was repeated (Figure 2b). Band shape for the 2,3-DHN changed markedly, suggesting that the EDTA wash had removed or masked the metal contaminants that were responsible for this problem. Whenever it is known that a particular sample compound complexes with heavy metals, and when the compound shows pronounced tailing that is not affected by TEA as modifier, EDTA can be used to wash the column (or the EDTA can be added to the mobile phase). Although other studies suggest that metal contamination of the column packing is not a general problem (12,13), the chelation and retention of sample compounds by the stainless-steel column frit can be important in some cases. Silanizing stainless-steel frits can help to avoid this problem or, alternatively, the use of stainless-steel screens in place of frits can effectively reduce metal-related problems (12).

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John W. Dolan is president of LC Resources Inc., of San Jose, California, and is a consulting editor for LC Magazine.