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# Slow equilibration of reversed-phase columns for the separation of ionized solutes

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**Abstract**

Reversed-phase columns that have been stored in buffer-free solvents can exhibit pronounced retention-time drift when buffered, low-pH mobile phases are used with ionized solutes. Whereas non-ionized compounds exhibit constant retention times within 20 min of the beginning of mobile phase flow, the retention of ionized compounds can continue to change (by 20% or more) for several hours. If mobile phase pH is changed from low to high and back again, an even longer time may be required before the column reaches equilibration at low pH. The speed of column equilibration for ionized solutes can vary significantly among different reversed-phase columns and is not affected by flow rate.


*Keywords:* Column equilibration; Ionized solutes

## 1. Introduction

Chromatographers often take column equilibration for granted. In some cases, the baseline will be monitored, until a constant detector signal is observed before injecting samples. Alternatively, the chromatographer may allow the mobile phase to flow through the column for a given time, followed by repetitive injections of a sample to ensure that retention times have become constant. Several studies of column equilibration in reversed-phase liquid chromatography (RP-LC) have been reported [1–6], none of which suggest that the time for column equilibration might vary with the nature of the sample. The present study began with the observation of unexpectedly long equilibration times for the retention of some solutes versus others. This is illustrated in Fig. 1, for the solute amitriptyline (a fully protonated strong base at pH 2.8). After an initial column equilibration of 20 min, the retention of amitriptyline continues to change for >10 h. This contrasts with the retention of neutral solutes, which typically remain constant after an initial equilibration period of 20 min. On the basis of the following discussion, we can recognize two kinds of column equilibration: a “fast” equilibration for neutral solutes and (in some cases) a “slow” equilibration for ionized compounds such as amitriptyline (which is independent of flow

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**Nomenclature**

| | |
|---|---|
| $k$ | solute retention factor |
| $k_{NB}$ | $k$ for nitrobenzene |
| $k_r$ | rate constant for slow column equilibration (Eq. (1)) |
| $k_0$ | value of $k$ at the beginning of slow column equilibration (Fig. 1) |
| $k_\infty$ | value of $k$ at the end of slow column equilibration (Fig. 1) |
| $Q$ | $(k - k_\infty)/(k_0 - k_\infty)$ (see Eq. (1)) |
| $R$ | $k_\infty/k_0$; the more $R$ deviates from 1.00, the greater are changes in $k$ during slow column equilibration |
| $R_s$ | resolution of two adjacent bands |
| S.D. | standard deviation |
| $t$ | time after the beginning of sample injections |
| $t_{1/2}$ | half-life of the slow column equilibration process; time in minutes for $(k - k_\infty)/(k_0 - k_\infty) = 0.5$ |
| –XH | an acidic group in the stationary phase that is presumed to ionize slowly, resulting in slow column equilibration (Eq. (3)) |

rate). Here, we are concerned only with "slow" column equilibration.

The example in Fig. 1 raises several questions concerning possible slow column equilibration:

- What is the cause of slow column equilibration (its physico-chemical basis)?
- How general and significant is slow column equilibration?
- How can the effects of slow column equilibration be anticipated and avoided?

Partial answers to these questions are proposed on the basis of work described here.

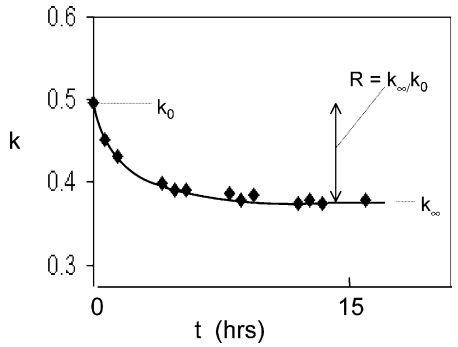


Fig. 1. Drift of amitriptyline retention with time after initial 20 min equilibration of column with mobile phase. Symmetry C18 column; 35 °C; 1.5 ml/min flow; mobile phase is 50% acetonitrile/buffer; buffer is 60 mM potassium phosphate, pH 2.8.

## 2. Experimental

### 2.1. Equipment, procedure and materials

The present study was carried out in two different laboratories (A and B). The equipment, procedures, and materials used in laboratory A have been described [7]: a Shimadzu HPLC system with auto-sampler, temperature control, and UV detection. Similar procedures and materials were used by laboratory B, except that the equipment consisted of a Model 1090 HPLC system with DAD detection (Hewlett-Packard) and a Merck column heater. Except where noted otherwise, the conditions used in these experiments were as follows: 15 cm × 0.46 cm columns of 5 μm $C_{18}$ packing from various suppliers; 50 vol.% acetonitrile/buffer mobile phase, the buffer consisting of 60 mM potassium phosphate in water with its pH adjusted prior to addition of acetonitrile, by combining 60 mM mixtures of phosphoric acid with monobasic potassium phosphate (for pH 2.8) or dibasic potassium phosphate (for pH 7.0), temperature of 35 °C; flow rate usually equal to 1.5 or 2.0 ml/min but specified in each case; UV detection at 205 nm.

### 2.2. Columns

Specific columns referred to here have been described previously (see Table 2 of [7]). For each column type (e.g. Symmetry C18), all experiments

reported here were carried out on columns from the same production lot.

### 2.3. Calculations

Values of $k$ were calculated from the retention $t_R$ and column dead-time $t_0$ as $(t_R - t_0)/t_0$, where $t_0$ is the retention time for thiourea.

## 3. Results and discussion

It has been assumed [3,4] that the mobile and stationary phases are in rapid equilibrium at every point within the column, so that column equilibration then requires the passage of a certain volume of mobile phase—rather than the passage of a certain time. This hypothesis plus experience suggests that 10–20 column volumes of mobile phase will usually suffice for column equilibration [8], except for mobile phases with a low organic content [1,6] or which contain an ion-pair reagent [9]. When using mobile phases similar to that of Fig. 1 (50% acetonitrile/buffer; buffer is 60 mM potassium phosphate at pH 2.8), neither are we aware of any previous report of a dramatic difference in equilibration times for different solutes, nor have we previously encountered a need for several hours of column equilibration.

As a means of simplifying the following discussion, we have summarized our various observations and their interpretation in Table 1. Table 1 suggests that, at low pH, some RP-LC columns experience a time-dependent change in charge (usually a decrease of the normal negative charge due to ionized silanols). As a result, the retention of ionized bases and other cations decreases with time, and that of ionized acids increases. We speculate that this change in column charge is due to an unspecified stationary-phase group $–X^-$ which slowly protonates at low pH to form –XH. The remainder of this paper deals with the details behind the observations and interpretations of Table 1.

### 3.1. Column equilibration as a function of column "history"

Successive equilibration experiments were carried out with a "virgin" (previously unused) Symmetry C18 column from the same production lot as in

Table 1
Summary of observations on slow column equilibration

| Variable | Observation | Interpretation |
|---|---|---|
| Solute structure | (1) Retention of cations and anions drifts in opposite directions (Table 3) | (1) Charge on column changes during slow equilibration |
| | (2) Extent of drift ($R$) increases for greater solute ionization (Table 3) | (2) Retention of partly ionized solute mainly due to the non-ionized molecule, which is not affected by slow equilibration |
| | (3) Rate of drift ($t_{1/2}$) similar for all ionized solutes (Table 3) | (3) Charge on column changes during slow equilibration |
| Column | (4) Extent and rate of drift varies significantly among different columns (Table 4 and Appendix A) | (4) The slow change in column charge is column dependent |
| Mobile phase pH | (5) Fast equilibration at pH 7.0, slow equilibration at pH 2.8 | (5) Relative change in column charge is small at pH 7.0, because the total change is larger |
| | (6) Exposure of column to high-pH or buffer-free mobile phase cancels column equilibration (Table 2b, Fig. 4) | (6) Column returns to initial "unequilibrated" condition (more negative column charge) |
| Temperature | (7) Higher temperatures provide faster column equilibration (smaller $t_{1/2}$) (Table 5) | (7) A temperature of 60 °C vs. 35 °C doubles the rate of slow column equilibration |
| Flow rate | (8) Slow column equilibration unaffected by flow rate (Table 5) | (8) Mobile phase composition does not change appreciably during slow column equilibration |
| Retention vs. time | (9) Eq. (1) obeyed | (9) Suggests 1st-order rate process for slow column equilibration |

Fig. 1. The column, which contains acetonitrile as solvent when received from the manufacturer, was connected to the HPLC system and flow of mobile phase (usually 50% acetonitrile/buffer, pH 2.8) was initiated (2.00 ml/min ≡ 1.3 column volumes/min). After 20 min of column equilibration, samples were injected at intervals of 10 min or longer until there was no change in retention times. The sample was a mixture of ethylbenzene, amitriptyline, and a quaternary-ammonium compound (berberine); the two latter solutes are completely ionized at pH 2.8. After completion of this series of injections, the column was then stored (usually in 50% acetonitrile/water) until the next day for repeating the column-equilibration experiment.

The results of the initial equilibration experiment with a virgin Symmetry C18 column are illustrated in Fig. 2 and summarized in Table 2a (experiment for "day 1"). Fig. 2a shows the change in $k$ for berberine with time, which matches a similar slow equilibration as in Fig. 1 for amitriptyline. Column equilibration in Fig. 2a appears to be described approximately by a first-order rate process. That is, given values of $k$ at the beginning ($k_0$) and end ($k_\infty$) of equilibration as defined in Fig. 1:

$$\log\left[\frac{k-k_\infty}{k_0-k_\infty}\right] \equiv \log Q = -k_r t \quad (1)$$

Here, $k_r$ is the rate constant and $t$ is time after the initial sample injection. The half-life time $t_{1/2}$ is given as:

$$t_{1/2} = \frac{0.301}{k_r} \quad (2)$$

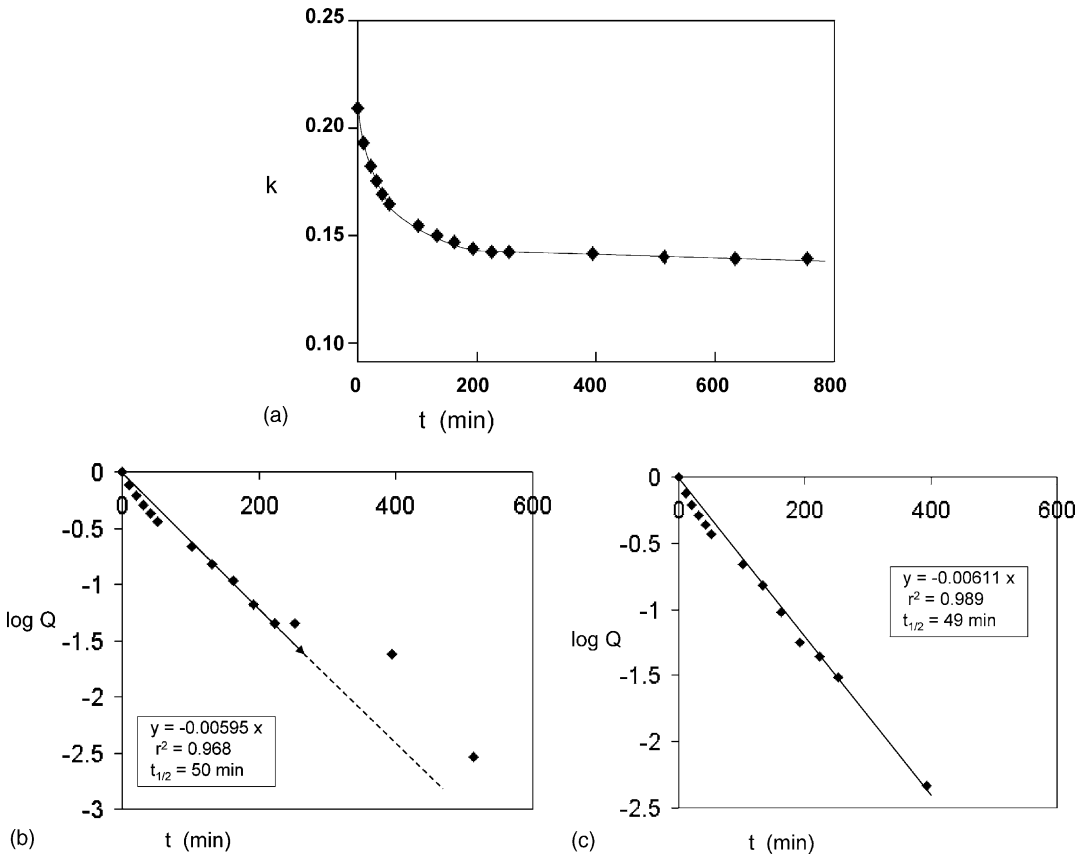


Fig. 2. Drift of berberine and amitriptyline retention with time; conditions as in Fig. 1, except 2.0 ml/min. (a) $k$ vs. time for berberine; (b) plot of Eq. (1) for data of (a); (c) plot of Eq. (1) for amitriptyline retention; $Q = (k-k_\infty)/(k_0-k_\infty)$.

Table 2
Effect of column storage on successive column equilibration cycles

| Column storage details (storage solvent precedes testing for each day) | Time | $t_{1/2}$ (min) | | $R$ | | $k_0$ | | $k_\infty$ | |
|---|---|---|---|---|---|---|---|---|---|
| | | Berb[a] | Ami[b] | Berb[a] | Ami[b] | Berb[a] | Ami[b] | Berb[a] | Ami[b] |
| (a) 100% acetonitrile (storage solvent) | | | | | | | | | |
| 100% acetonitrile | Day 1 | 50 | 49 | 0.67 | 0.76 | 0.209 | 0.468 | 0.140 | 0.356 |
| 50% acetonitrile/water for 12 h | Day 2 | 49 | 51 | 0.81 | 0.86 | 0.198 | 0.452 | 0.160 | 0.389 |
| 50% acetonitrile/water for 42 h | Day 4 | 66 | 66 | 0.76 | 0.83 | 0.216 | 0.477 | 0.165 | 0.396 |
| 100% acetonitrile for 11 h | Day 5 | 55 | 52 | 0.73 | 0.80 | 0.240 | 0.517 | 0.176 | 0.414 |
| | Average | 55 ± 8 | 55 ± 8 | 0.74 ± 0.06 | 0.81 ± 0.04 | – | – | – | – |
| (b) 50% acetonitrile/pH-7.0 buffer for 6 h | | | | | | | | | |
| First in 100% acetonitrile, then in 50% acetonitrile/buffer for 6 h | Day 6 | 72 | 70 | 0.64 | 0.70 | 0.441 | 0.830 | 0.283 | 0.584 |
| In mobile phase for 48 h | Day 8 | 56 | 51 | 0.87 | 0.90 | 0.232 | 0.501 | 0.201 | 0.453 |
| In 100% acetonitrile for 43 h | Day 10 | 86 | 81 | 0.77 | 0.80 | 0.248 | 0.559 | 0.192 | 0.448 |
| In mobile phase for 60 h | Day 13 | 0[c] | 0[c] | 0.99 | 0.99 | 0.170 | 0.404 | 0.168 | 0.401 |
| (c) Brief (1 h) storage in 50% acetonitrile/water | | | | | | | | | |
| In 50% acetonitrile/water for 1 h | Day 13-1 | 21 | 22 | 0.82 | 0.86 | 0.221 | 0.489 | 0.181 | 0.423 |
| | Day 13-2 | 22 | 20 | 0.82 | 0.87 | 0.225 | 0.496 | 0.186 | 0.430 |
| | Day 13-3 | 23 | 22 | 0.81 | 0.87 | 0.230 | 0.501 | 0.187 | 0.435 |

Conditions involve a single Symmetry C18 column, 50% acetonitrile/buffer (pH 2.8) mobile phase, 35 °C and 2.0 ml/min and equilibration with mobile phase for 20 min prior to sample injections. The $t_{1/2}$ values in part (c) are approximate, due to incomplete column equilibration during each equilibration of the column.

[a] Berberine.

[b] Amitriptyline.

[c] Column nearly equilibrated at the beginning, so $t_{1/2}$ not calculated.

The data of Fig. 2a are replotted in Fig. 2b according to Eq. (1), showing an approximately linear dependence of log $Q$ on time, as required by a first-order rate process. A similar plot for amitriptyline in this first equilibration experiment ("day 1") is shown in Fig. 2c.

The retention of the neutral solute ethylbenzene during the experiments in Fig. 2 was $k = 9.54 \pm 0.02$ min (S.D. = 1); i.e. constant over the initial 2 h of sample injections. The initial 20 min flow of mobile phase through the column appears to have resulted in an equilibrated column for ethylbenzene and other neutral solutes ("fast" equilibration), whereas this is clearly not the case for berberine and amitriptyline ("slow" equilibration). Further equilibration experiments were carried out as in Fig. 2, where each series of injections was performed on a different day. The results of 4 days of such experiments are summarized in Table 2a. Similar values of $t_{1/2}$ were observed on each day for each solute: $t_{1/2} = 55 \pm 8$ min (S.D. = 1). Likewise, the extent of retention drift (i.e. change in $k$ in one direction) as measured by values of $R = k_\infty/k_0$ were similar on all days, although lower for berberine ($R = 0.74 \pm 0.06$) compared to amitriptyline ($R = 0.81 \pm 0.04$). Values of $k_0$ and $k_\infty$ tend to rise slightly every day; Fig. 3 shows a plot of $k_\infty$ for amitriptyline from day 1 to day 5 (diamonds).

The experiments outlined in Table 2a were followed by the experiments of Table 2b ("day 6" to "day 13"). Prior to the experiment of day 6, the column was stored for 6 h with mobile phase that had been adjusted to pH 7.0, after which the column was returned to mobile phase pH 2.8 during these experiments. The usual column equilibration experiments at pH 2.8 were then followed for subsequent days (summarized in Table 2b). These are similar to the experiments of Table 2a, except that in some cases, the column was stored in mobile phase pH 2.8 between experiments. The most significant consequence of this high-pH column treatment is seen in values of $k_\infty$ for the two solutes; i.e. for retention with the equilibrated column. For both solutes, values of $k_\infty$ increase immediately after the high-pH treatment ("day 6", 0.283 and 0.584 for berberine and amitriptyline, respectively),

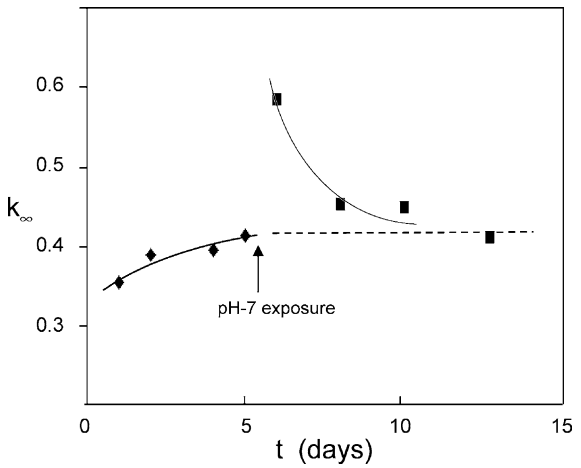


Fig. 3. Values of $k_\infty$ for amitriptyline vs. time; data of Table 2a and b. Column was filled with pH 7.0 mobile phase for 6 h between days 5 and 6 (see text for details).

followed by a very gradual return on days 8 and 10, towards values of $k_\infty$ observed on day 5 as in Table 2a (0.176 and 0.414, respectively, immediately prior to exposure of the column to pH 7.0). Fig. 3 for the amitriptyline experiments shows post-pH 7.0 results (squares), which suggest a final (fully equilibrated) value of $k_\infty \approx 0.41$ after day 13. That is, after equilibration of the column as in Fig. 1a, there is a further very gradual and less pronounced change in the values of $k_\infty$ over a period of several days. The day-to-day change in retention for an equilibrated column is of somewhat less practical significance (compared to slow column equilibration within the same day) and would be overlooked in most cases. The average values of $k$ for ethylbenzene remained essentially the same from day 1 ($9.54 \pm 0.02$) to day 10 ($9.64 \pm 0.04$).

When an equilibrated column is exposed to unbuffered mobile phase for a short time, it returns to its unequilibrated condition fairly rapidly. This is illustrated by the data of Table 2c, where three column equilibration experiments were carried out, interspersed by 1 h of conditioning the column with 50% acetonitrile/water. Values of $k$ versus time for amitriptyline in these experiments are plotted in Fig. 4.

### 3.2. Column equilibration as a function of solute structure

Using the Symmetry C18 column, approximate values of $R$ and $t_{1/2}$ (due to incomplete data) were recovered from the study of [7] for several ionizable solutes (acids and bases) and neutral compounds, as summarized in Table 3. Similar values of $R$ ($0.78 \pm 0.05$) and $t_{1/2}$ ($78 \pm 16$) are observed for all five (fully protonated) strong bases. The two moderately strong acids (37–55% ionized) also exhibit similar values of $t_{1/2}$

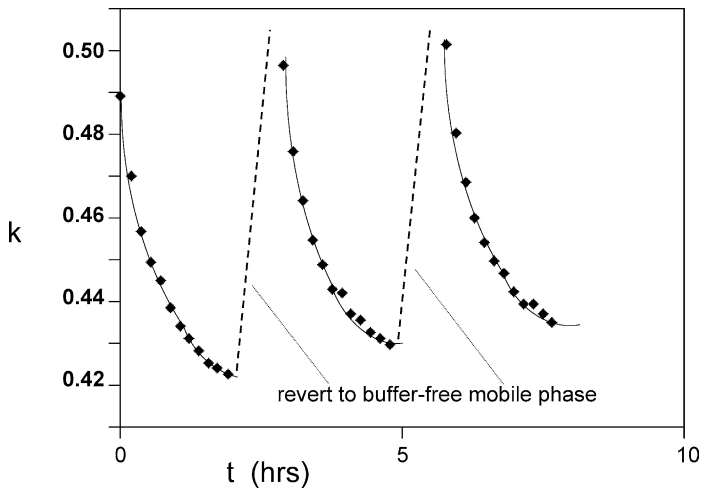


Fig. 4. Effect of intermittent conditioning of the column with 50% acetonitrile/water [pH-2.8] for 1 h, followed by partial equilibration of the column with 50% acetonitrile/buffer for 2 h (repeated three times) (see Table 2c and related discussion).

Table 3
Slow column equilibration as a function of the sample

| Solute | $k_\infty$ | $R$ | Charge[a] | $t_{1/2}$ (min) |
|---|---|---|---|---|
| Strong bases (fully protonated) | | | | |
| Amitriptyline | 0.4 | 0.82 | 1 | 88 |
| Prolintane | 0.2 | 0.74 | 1 | 63 |
| Diphenhydramine | 0.2 | 0.74 | 1 | 61 |
| Propranolol | 0.1 | 0.74 | 1 | 80 |
| Nortriptyline | 0.3 | 0.83 | 1 | 97 |
| Average | – | 0.78 ± 0.05 | 1 | 78 ± 16 |
| Moderately strong acids | | | | |
| 2-Nitrobenzoic acid | 0.3 | 1.05 | −0.55 | 95 |
| Diflunisal | 2.7 | 1.11 | −0.37 | 78 |
| Average | – | 1.08 ± 0.04 | −0.46 | 86 ± 12 |
| Weak acids | | | | |
| 2,6-Dimethylbenzoic acid | 0.9 | 1.00 | −0.08 | – |
| 3-Cyanobenzoic acid | 0.5 | 1.00 | −0.09 | – |
| 3-Nitrobenzoic acid | 0.4 | 1.00 | −0.10 | – |
| 2-Fluorobenzoic acid | 0.6 | 1.02 | −0.08 | – |
| Ketoprofen | 2.3 | 1.02 | −0.01 | – |
| Mefenamic acid | 10.2 | 1.02 | −0.02 | – |
| 4-*n*-Butylbenzoic acid | 5.1 | 1.02 | −0.01 | – |
| 4-*n*-Pentylbenzoic acid | 8.6 | 1.04 | 0.00 | – |
| 4-*n*-Hexylbenzoic acid | 14.8 | 1.04 | 0.00 | – |
| Average | – | 1.02 | −0.04 | – |
| Weak bases | | | | |
| 2-Phenylpyridine | 1.6 | 1.00 | 0.61 | – |
| *N*-Ethylaniline | – | 1.04 | 0.90 | – |
| 4-*n*-Pentylaniline | – | 1.00 | 0.71 | – |
| 4-*n*-Hexylaniline | – | 1.00 | 0.71 | – |
| 4-*n*-Heptylaniline | – | 1.00 | 0.71 | – |
| Average | – | 1.01 | 0.73 | – |
| Neutral compounds | | | | |
| Nitrobenzene | – | 1.00 | 0.00 | – |
| Benzotrichloride | – | 1.00 | 0.00 | – |

Symmetry C18 column, 50% acetonitrile/buffer (pH 2.8), 35 °C, 1.5 ml/min. Approximate values derived from limited data.
[a] Molecular charge, as estimated in [10]; "1.00" means 100% ionized.

(86±12), but have values of $R > 1$. That is, these acids show an increase in retention as column equilibration proceeds, but the overall change in average retention is smaller (+8% versus −22% for the strong bases). Weak acids, bases, and neutrals show values of $R \approx 1$; i.e. little or no slow column equilibration effects. Estimates of solute molecular charge in Table 3 (third column of data) were obtained in [10] from retention in the same mobile phase (50% acetonitrile/buffer) as a function of pH, using the Henderson–Hasselbach equation.

### 3.3. Column equilibration as a function of the column

The experiments of Table 3 were repeated for nine other columns; results for amitriptyline as solute are summarized in Table 4. For each column, results for the other solutes of Table 3 were generally consistent with data for amitriptyline, i.e. if significant retention drift occurred for amitriptyline, other strong bases also experienced significant drift, while partly-ionized acids drifted to a lesser extent in the opposite

Table 4
Slow column equilibration as a function of the column

| Column | $k_\infty$ | $R$ | $t_{1/2}$ (min) | Eq. (1) applies? | CV for $k_{NB}$[a] (%) |
|---|---|---|---|---|---|
| Symmetry C18 | 0.4 | 0.82 | 88 | Yes | 0.16 |
| YMC 15[b] | 0.5 | 0.78 | 119 | Yes | 0.11 |
| YMC 16[b] | 0.6 | 0.81 | 125 | Yes | 0.17 |
| YMC 17[b] | 0.6 | 0.81 | 175 | Yes | 0.16 |
| Supelco discovery C18 | 0.5 | 0.96 | 114 | Yes | 0.14 |
| Eclipse XDB C18 | 0.6 | 0.98 | – | No | 0.20 |
| Inertsil ODS-3 | 0.4 | 0.98 | – | No | 0.15 |
| StableBond 300A C18 | 0.3 | 1.03 | – | No | 0.13 |
| StableBond 80A C18 | 0.7 | 1.01 | – | No | n.d. |
| StableBond 80A C18[c] | 0.7 | 1.05 | – | No | – |

Data for amitriptyline as solute unless noted otherwise, other conditions as in Table 3. Approximate values derived from limited data.
[a] Variation of $k$ for nitrobenzene ($k_{NB}$) during slow column equilibration.
[b] Different lots of YMC ProPack C18, varying in carbon percentage (see Table 2 of [1] for details).
[c] Column intentionally has 10% less bonded phase than preceding column.

direction. If amitriptyline did not experience significant retention drift, no other solute did.

For the first five columns of Table 4 (including symmetry C18), a generally similar behavior was found for amitriptyline. Changes in retention during column equilibration appeared to follow first-order kinetics (Eq. (1)) with values of $R < 1$. Values of $R$ were similar (0.78–0.82) for the first four columns, while values of $t_{1/2}$ ranged from 88–175 (note that the three YMC columns of Table 4 are from different lots of YMC ProPack C18). The last five columns of Table 4 exhibited a quite different behavior; values of $R$ were close to 1.00 or exceeded 1.00 (i.e. the retention of amitriptyline increased slightly with time for the last column of Table 4). Values of $k$ appeared to change linearly with time for these five columns, rather than according to Eq. (1).

Limited experiments with a Symmetry C8 column (data not reported) gave results similar to those for Symmetry C18, except for a reversal of the direction of retention drift (values of $k_\infty > k_0$ for basic solutes and $k_\infty < k_0$ for acidic solutes); i.e. the retention of bases increased with time, while acidic solutes showed decreased retention with time.

### 3.4. Column equilibration as a function of separation conditions

#### 3.4.1. Mobile phase pH

The experiments of Table 2b and Fig. 3 show that exposure of the column to pH 7.0 mobile phase results in an increase of $k_\infty$, presumably due to an increase in the negative charge on the column. Other experiments have established that column equilibration is

Table 5
Effect of other separation variables on slow column equilibration

| Time | Variable | Change | $R$[a] | $t_{1/2}$[a] (min) | $k_\infty$[b] |
|---|---|---|---|---|---|
| Day 1 | – | No change | 0.72 | 44 | 0.445 |
| Day 2 | Temperature | 35 → 60 °C | 0.90 | 20 | 0.466 |
| Day 5 | Percentage acetonitrile | 50 → 30% | 0.91 | 57 | 7.9 |
| Day 6 | Temperature and percentage acetonitrile | 35 → 60 °C, 50 → 30% | 0.94 | 22 | 5.8 |
| Day 7 | – | No change | 0.81 | 41 | 0.565 |
| Day 8 | Flow rate | 2.0 → 0.5 ml/min | 0.84 | 46 | 0.407 |

Symmetry C18 column, amitriptyline and berberine solutes. Conditions are the same as in Table 2, except where noted otherwise.
[a] Average of values for amitriptyline and berberine.
[b] For amitriptyline.

much faster for high-pH mobile phases. Thus, for a mobile phase pH 7.0, the equilibration of the column for amitriptyline is complete within a time $t < 1$ h [11]; i.e. $t_{1/2} < 10$ min.

### 3.4.2. Other separation conditions

Additional experiments were carried out with a new ("virgin") Symmetry C18 column as summarized in Table 5. The initial experiment ("day 1") demonstrated the usual slow column equilibration, yielding similar average values of $R$ and $t_{1/2}$ for berberine and amitriptyline ($R = 0.72$, $t_{1/2} = 44$) as were found in Table 2a for another column from the same production batch ("day 1", $R$ (average of 0.67 and 0.76)= 0.715, $t_{1/2}$ (average of 49 and 50)= 49.5). In successive experiments on days 2–7 of Table 5, various separation conditions were changed and values of $R$ and $t_{1/2}$ determined. Thus, on day 2, the temperature was changed from 35 to 60 °C; on day 5, the acetonitrile percentage in the mobile phase was changed from 50 to 30%; on day 6, both temperature and acetonitrile percentage were changed, and on day 7 the original experiment of day 1 was repeated, followed by a change in flow rate. Between these various experiments, the column was stored in 50% acetonitrile/water.

The results for days 1 and 7 (no change in conditions) are similar to those for identical experiments on a different virgin column: average value of $R = 0.78$ (Table 2a) and 0.77 (Table 5); average value of $t_{1/2} = 55$ (Table 2a) and 41 (Table 5). The effect of an increase in temperature is an increase in the rate of column equilibration ($t_{1/2} = 20$) and an apparent reduction in the change in retention during equilibration ($R = 0.92$). However, the latter reduction in $R$ is largely accounted for, by the faster column equilibration during the 20 min period prior to the first sample injection. A lower acetonitrile percentage provides $R = 0.91$, with possibly a slight slowing down of column equilibration ($t_{1/2} = 57$).

The effect of a change in flow rate from 2.0 to 0.5 ml/min was measured directly, as summarized in Table 5, day 8. Similar values of $R$ (= 0.81, 0.84) and $t_{1/2}$ (= 41, 46) were obtained for both flow rates, confirming that slow column equilibration is not significantly affected by change in flow rate; i.e. static equilibration (no flow) should be equivalent to flow equilibration. This was confirmed in a previous study [11], where a static equilibration of the Symmetry C18 column with the same pH 2.8 mobile phase for 16 h (prior to replicate sample injections) resulted in constant values of $k$ for amitriptyline: $k = 0.363 \pm 0.003$ (S.D. = 1) over a 9 h period column equilibration.

## 3.5. The physico-chemical basis of slow column equilibration

The present study as summarized in Table 1, strongly suggests that slow column equilibration at low pH is a consequence of a slow change in the charge on the column during equilibration. The cause of varying column charge during slow column equilibration is at present unknown, although one possibility is a slow diffusion of protons between the mobile phase and restricted regions of the stationary phase.

The column normally carries a sizable negative charge at pH $\geq$ 6 and a smaller negative charge at lower pH, due to the presence of two different kinds of ionizable silanols [12,13]. The equilibration of silanols with the adjacent mobile phase has been presumed fast, as is usually the case for acid–base reactions in solution. The much slower change in column charge during slow equilibration is atypical, hence differentiating the ionizable column groups (–XH) responsible for slow-equilibration from "normal" silanols (–SiOH).

This change in column charge is in most cases positive; i.e. the column becomes less negative during slow equilibration:

$$-X^- + H^+ \rightarrow XH \quad (3)$$

The latter hypothesis explains the drift of solute retention in different directions for cationic and anionic solutes; i.e. as the column loses negative charge, its attraction for cations decreases, while anions are retained more strongly (Table 1, #1). Solutes that are only partially ionized exhibit smaller changes in retention during slow column equilibration, because their retention is mainly due to the non-ionized molecule (Table 1, #2). The only group of solutes in Table 3, which appear out of line are the weak bases. Despite having an average molecular charge of +0.73 at pH 2.8; values of $R \approx$ zero within experimental error, in other experiments, however, weak bases did exhibit slow column equilibration, albeit to a lesser extent. The rate of retention drift as measured by values of $t_{1/2}$ is roughly the same for different solutes (Table 1,

#3) and a given column, which also agrees with a slow change in column charge during equilibration.

The fact that the rate and extent of retention drift vary among different columns (Table 1, #4), indicates that (a) the concentration of the slow-equilibration species –XH varies from one column to another, and (b) the rate of the reaction of Eq. (3) also varies. For columns that undergo slow equilibration at low pH, column equilibration is much faster at pH 7.0 (Table 1, #5); i.e. no appreciable slow column equilibration. This can be rationalized by assuming that the concentration of the group –XH (or $–X^-$) is much smaller than that of ionized silanols at pH 7.0, so that relative changes in column ionization with time due to the ionization of –XH at pH 7.0 will be quite small. Consequently, changes in the relative retention of ionized solutes will also be small. Since silanol ionization for type-B columns is much reduced at pH 2.8 [12,13], changes with time in the ionization of –XH lead to larger changes in the relative charge on the column, with a larger effect on solute retention.

When the column is exposed to mobile phase without buffer (i.e. neutral pH $\approx$ 7.0), the low-pH equilibration of the column is negated (Table 1, #6); i.e. $XH \rightarrow X^-$ as prior to column equilibration. Exposure of the column to higher pH, followed by low-pH equilibration, gives a qualitatively similar result as for a buffer-free mobile phase, presumably for similar reasons.

The exposure of an alkyl-silica column to low-pH conditions for an extended period can result in partial loss of the bonded phase due to cleavage of the Si–O–Si– bond [15]. This effect cannot be used to explain the reversible slow column equilibration observed here. However, the extended equilibration of alkyl-silica columns at low pH should be done with care, in view of the possible loss of bonded phase (especially in the case of trimethylsilyl end-capping, which is especially easily lost at low pH [15]).

### *3.6. Practical implications of slow column equilibration*

#### *3.6.1. How widespread and significant is slow column equilibration?*

The present study offers several reasons why slow column equilibration might have been overlooked in the past. First, unless the sample contains ionized solutes, slow column equilibration has no observable consequences. Second, fully ionized solutes will usually be less retained (smaller $k$), so that significant relative changes in $k$ will translate into smaller changes in absolute retention time—and therefore be less noticeable. Third, slow column equilibration does not occur with all columns. Fourth, the effects of slow column equilibration are considerably reduced at higher temperatures. Finally, RP-LC separations in the past were often carried out using mobile phases with pH $>$ 5, in which case, column equilibration should be fast for all solutes and columns. However, with the recognition that basic solutes are more likely to tail at higher pH [14], chromatographers today are more likely to use low-pH conditions for the separation of these compounds, and are therefore more likely to experience slow column equilibration.

Four out of 10 columns in Table 4 (as well as Symmetry C8) have values of $R \approx 0.8$, and are therefore significantly affected by slow column equilibration. Semi-quantitative evidence from our laboratory suggests that 40% of all columns currently sold may be subject to some degree of slow column equilibration (see Appendix A).

#### *3.6.2. How can the effects of slow column equilibration be avoided?*

Changes in sample retention as a result of slow column equilibration are more likely, if the column is exposed to high-pH conditions prior to separations at low pH. Dedication of the column to a single set of conditions (or for a given assay procedure) can eliminate retention changes due to a change in pH. Note also that slow column equilibration is a function of time, rather than the volume of mobile phase that has passed through the column. We have shown previously [11] that a static equilibration of the column with mobile phase prior to initiating flow of mobile phase through the column can eliminate slow column equilibration. Alternatively, if the column is always stored in the mobile phase, the column remains in an equilibrated state.

## 4. Conclusions

As many as four out of 10 RP-LC columns may be subject to “slow column equilibration”, whereas for low-pH mobile phases and samples that contain

ionized solutes, 5 h or more of column equilibration may be required before solute retention times become constant (±1%). The problem of slow column equilibration appears to involve small changes in the negative charge on the column, which is otherwise due to ionized silanols. As a result, the retention of cationic and anionic solutes drifts in opposite directions. The cause of slow column equilibration has not been determined.

It has been established that slow column equilibration does not depend on flow rate. Therefore, column equilibration can be conveniently effected by storing the column in mobile phase prior to use. Slow column equilibration is not likely to represent a significant problem for most assay procedures. The column is often equilibrated for 1 h or 2 h prior to sample injection, and further changes in sample retention with use are likely to be small. However, for samples and separation conditions that are conducive to slow column equilibration (ionized sample molecules, pH $< 6$, temperatures $<40\,^{\circ}C$), it is recommended that experiments be carried out to first establish the magnitude of the effect (as in Fig. 1). For theoretical studies that require highly precise retention times (e.g. changes in $k \leq 2\%$), slow column equilibration represents a potential problem that should be considered in the design of related experiments.

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## Appendix A. A study of slow column equilibration for 19 different RP-LC columns

We subjected 19 different type-B columns from eight different manufacturers (including only three of the columns of Table 4) to tests which involved initial injections of amitriptyline at pH 2.8 (same mobile phase as in Table 2) over a 50 min interval which followed an approximate 20 min equilibration cycle. The column was next exposed to pH 7.0 for about 1 h, followed by an approximate 20 min equilibration with pH 2.8 mobile phase and subsequent injection of amitriptyline. We determined changes in retention during initial injections at pH 2.8 (i.e. slow column equilibration, expressed as percentage change in $k$), and the change in values of $k_0$ before and after exposure of the column to pH 7.0. In the absence of slow column equilibration, we would expect little retention drift at pH 2.8 and only minor changes in values of $k_0$ before and after the column was exposed to pH 7.0.

For 11 of the 19 columns, the column appeared to equilibrate quickly at pH 2.8, both before and after exposing the column to pH 7.0: the average change in $k$ during elution with pH 2.8 mobile phase for 50 min was $-0.4 \pm 0.8\%$ (S.D. $= 1$); average change in $k_0$ after pH 7.0 exposure was $-0.3 \pm 3.5\%$. These 11 columns do not appear to be subject to slow column equilibration as described above.

For the remaining eight columns, significantly larger changes in $k$ were observed: average change in $k$ during elution for 50 min with pH 2.8 mobile phase equal $-4.0 \pm 1.2\%$; average change in $k_0$ before and after pH 7.0 exposure equal $-6 \pm 29\%$ (i.e. both large positive and negative changes were observed). These results suggest that many columns (as well as some lots of a given column) will experience significant retention drift during slow column equilibration; even larger changes in retention can be experienced for changes in mobile phase pH. On the basis of results presented here, it appears that about 40% of all commercial columns may experience some degree of slow equilibration.

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