# Re-equilibration in HILIC Chromatography

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## Introduction

Interest in chromatography using hydrophilic interaction liquid chromatography (HILIC) has continued to build in recent years. Full adoption of the technique, however, has been slowed by experiences of poor reproducibility. Reproducibility issues generally stem from a lack of understanding of the controls one sets up in a chromatographic system. Re-equilibration times in HILIC, for example, have been reported as being exceptionally long as compared to reversed-phase chromatography. Is it possible, then, that some reproducibility issues are a result of improper re-equilibration settings?

In this study, re-equilibration times in HILIC, for both aqueous:organic gradients and buffer gradients are systematically explored. Repeatability of retention and selectivity was studied as a function of equilibration times following aqueous:organic gradients.

The impact of the use of buffer gradients and subsequent equilibration procedures on retention times were also investigated using several HILIC stationary phases and probes. The results not only promise to improve method development practices, but also provide valuable insight into HILIC retention mechanisms across a diverse set of polar stationary phases.

## Experimental

### Organic: Aqueous Gradient Study

Using a bare silica (Ascentis Express HILIC, 10 cm x 3.0 mm, 2.7 µm) and a pentahydroxy phase (Ascentis Express OH5, 10 cm x 3.0 mm, 2.7 µm), a set of neutral molecules was run employing a gradient from 5% aqueous to 50% aqueous with varied equilibration times. The same study was also performed using a gradient from 5% to 25% of the aqueous component. Mobile phase A was 5 mM ammonium acetate in 95% acetonitrile while mobile phase B was 5 mM ammonium acetate in 50% acetonitrile. Mobile phase C was 5 mM ammonium acetate in 75% acetonitrile. Gradient 1 ran from A to B in 10 minutes. Gradient 2 ran from A to C in 10 minutes. Re-equilibration times were set at 2, 5, 10, 15 and 20 minutes. The column temperature was held at 35 ºC, flow rate at 0.5 mL/min, injection Volume at 2 µL and detection by UV absorbance at 254 nm. Initially, a set of neutral polar analytes (adenosine and cytosine) were injected in triplicate. To represent polar basic molecules, metanephrine and normetanephrine were also tested.

### Buffer Gradient Study

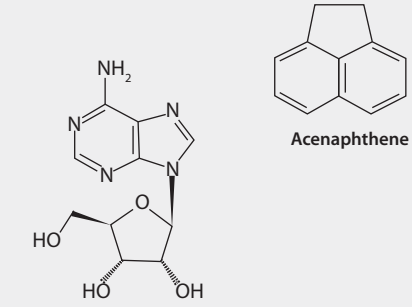
The buffer gradient study was run identically to the organic:aqueous study except that mobile phase A consisted of 2 mM ammonium acetate in 90% acetonitrile, pH* adjusted to 7.0 (+/- 0.05) with acetic acid and mobile phase B consisted of 10 mM ammonium acetate in 90% acetonitrile, pH* adjusted to 7.0 (+/- 0.05) with acetic acid (pH measured in the presence of organic and calibrated using aqueous standards). The gradient was run from 100% A to 100% B in 10 minutes. In addition to the polar neutral mix and the polar basic mix, an additional nonpolar basic mix was included.

## Results and Discussion

Figure 1 shows the structures of the test probes used throughout the study. Adenosine and cytosine represent neutral polar molecules that should only interact with the stationary phase via partition or polar (dipole type) interactions. The metanephrines are both polar and positively charged under the conditions of the study and thus can interact via partitioning, polar and ionic mechanisms. Lastly, the tricyclic antidepressants (TCAs) amitriptyline and nortriptyline are ionized, but relatively nonpolar. The TCAs, then, are expected to retain via ion-exchange mechanisms and perhaps some polar interactions, but not via partition.

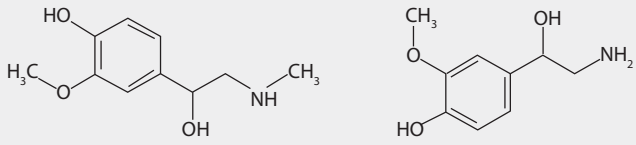
**Figure 1. Structures of the Study Probe Molecules**

**Polar Neutral**



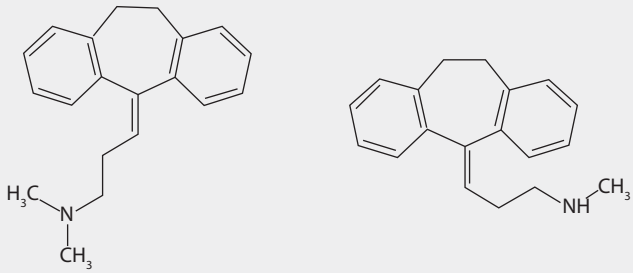

Adenosine [*{BAN}; *{USAN}] Cytosine

**Polar Basic**




Metanephrine Normetanephrine

**Nonpolar Basic**




Amitriptyline Nortriptyline

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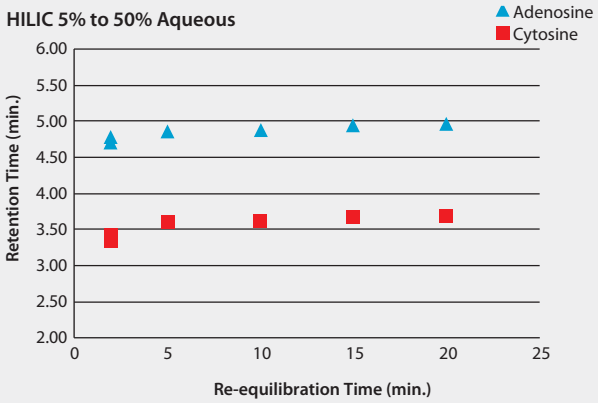
Plots of retention time versus organic-aqueous re-equilibration time are shown in **Figure 2** for the neutral probes. For the steeper gradient (to 50% aqueous) there exists some slight irreproducibility at 2 minutes re-equilibration time, however at 5 minutes and above, highly reproducible results are obtained. For the shallower gradient just two minutes appears to be sufficient to generate reproducible results. In both plots the retention drifts upwards as re-equilibration times increase, however at each given time point, reproducible results are obtained.

**Figure 2. Retention as a Function of Re-equilibration Time**



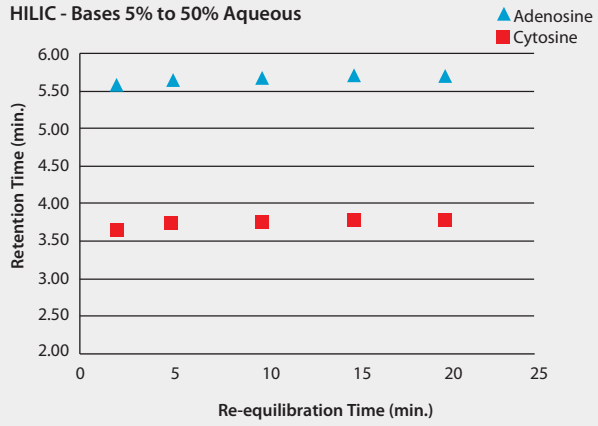




**Figure 3. Retention as a Function of Re-equilibration Time**

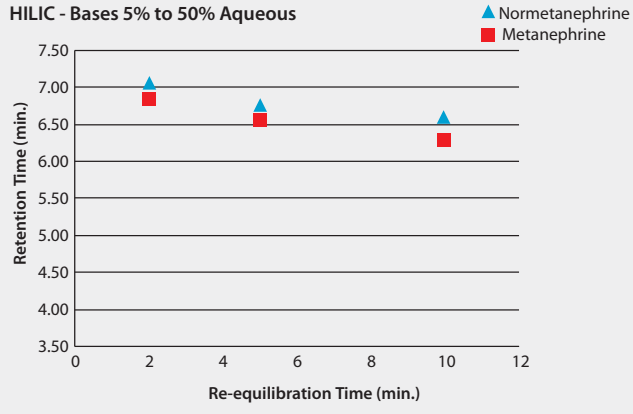
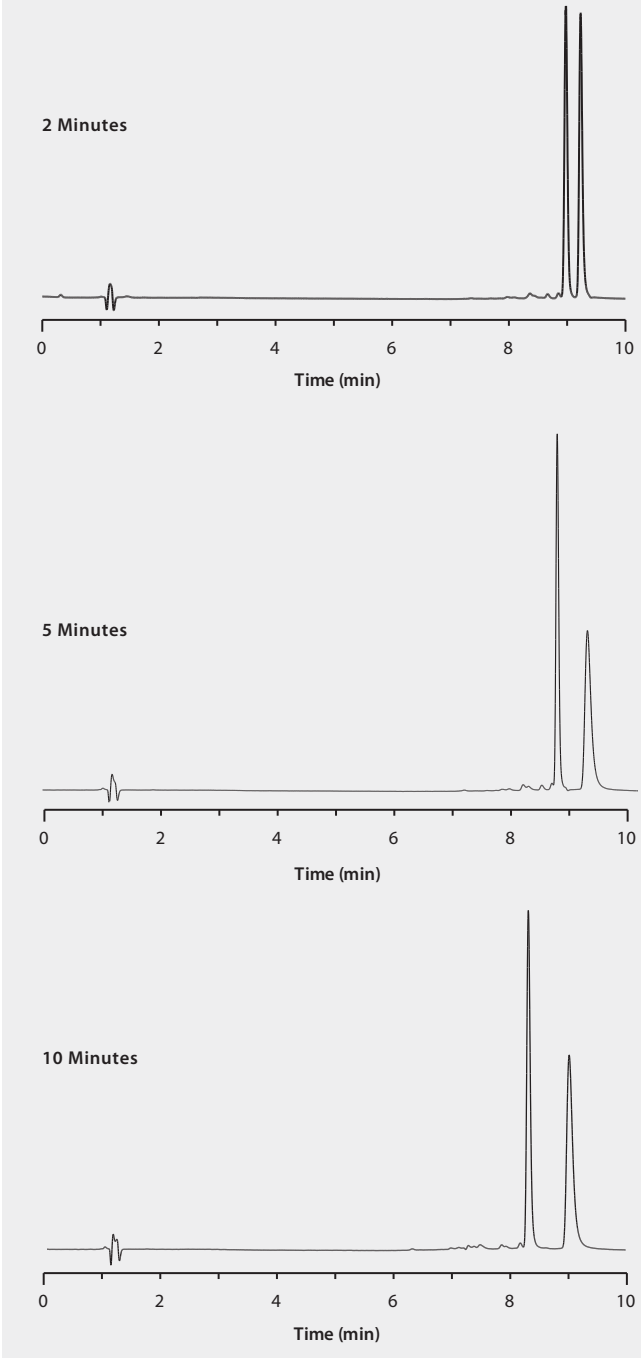



**Figure 3** shows the results from the polar basic probes using the steep gradient on the HILIC phase. Again the retention times are shown to be highly reproducible at a given re-equilibration time. In the case of the polar bases the retention drifts downward as a function of re-equilibration time. In addition, as shown in **Figure 4**, selectivity and peak shape for the polar bases are significantly impacted by the re-equilibration time on the bare silica phase.

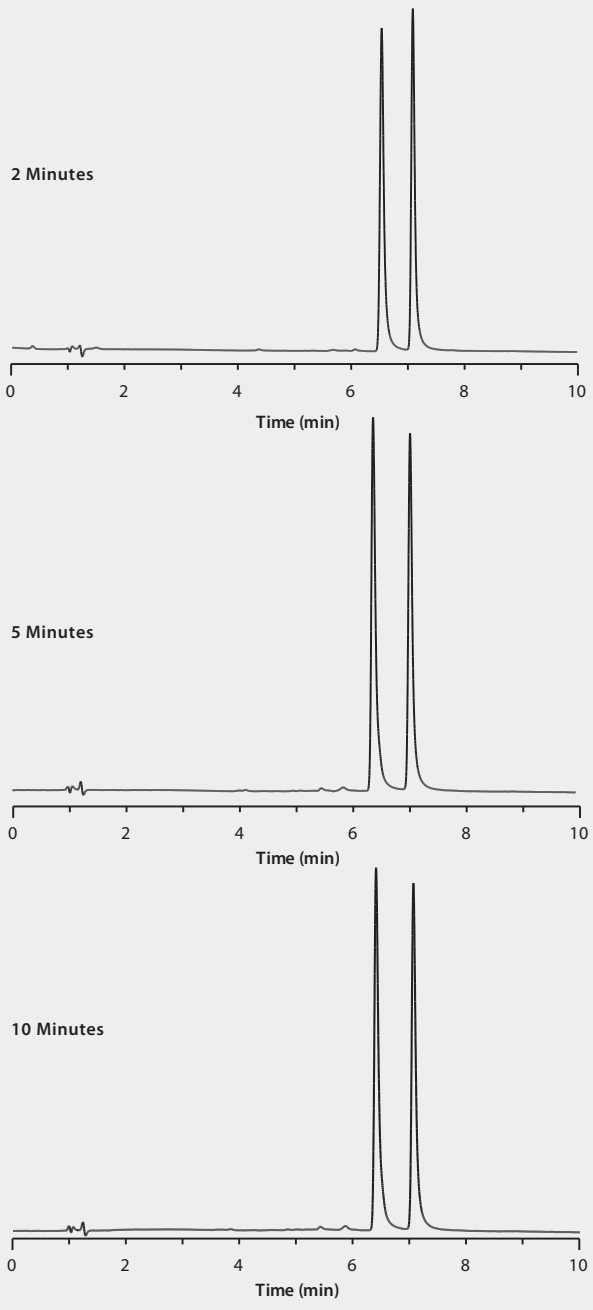
**Figure 4. Chromatographic Traces of Basic Analytes on HILIC Phase at Different Re-equilibration Times**



The chromatographic traces obtained for the polar bases on the OH5 phase are shown in **Figure 5**. In this case, the re-equilibration times do not appear to significantly impact the retention, selectivity or peak shape.

In previous studies (ref HILIC webinar?), the OH5 phase has shown less ion-exchange character as compared to bare silica HILIC phases. This observation coupled with the faster equilibration of the basic analytes suggests that ion-exchange mechanisms are the main cause of slow equilibration on the bare silica phase. To investigate this further buffer gradients were run on both phases and an additional set of nonpolar, basic probes was added.

**Figure 5. Chromatographic Traces of Basic Analytes on OH5 Phase at Different Re-equilibration Times**




**Figures 6** and **7** show the plots of retention times versus buffer gradient re-equilibration times for each set of test probes on the bare silica and pentahydroxy phases, respectively. On both phases, the neutral polar molecules are barely affected. For both the polar and nonpolar bases, retention increases as re-equilibration time increased. The magnitude of the increase on the OH5 phase, however, is greatly attenuated as compared to the bare silica phase for both sets of basic compounds. It is also important to note that reproducibility at any given re-equilibration time on both phases was observed.

**Figure 6. Retention as a Function of Re-equilibration Time**

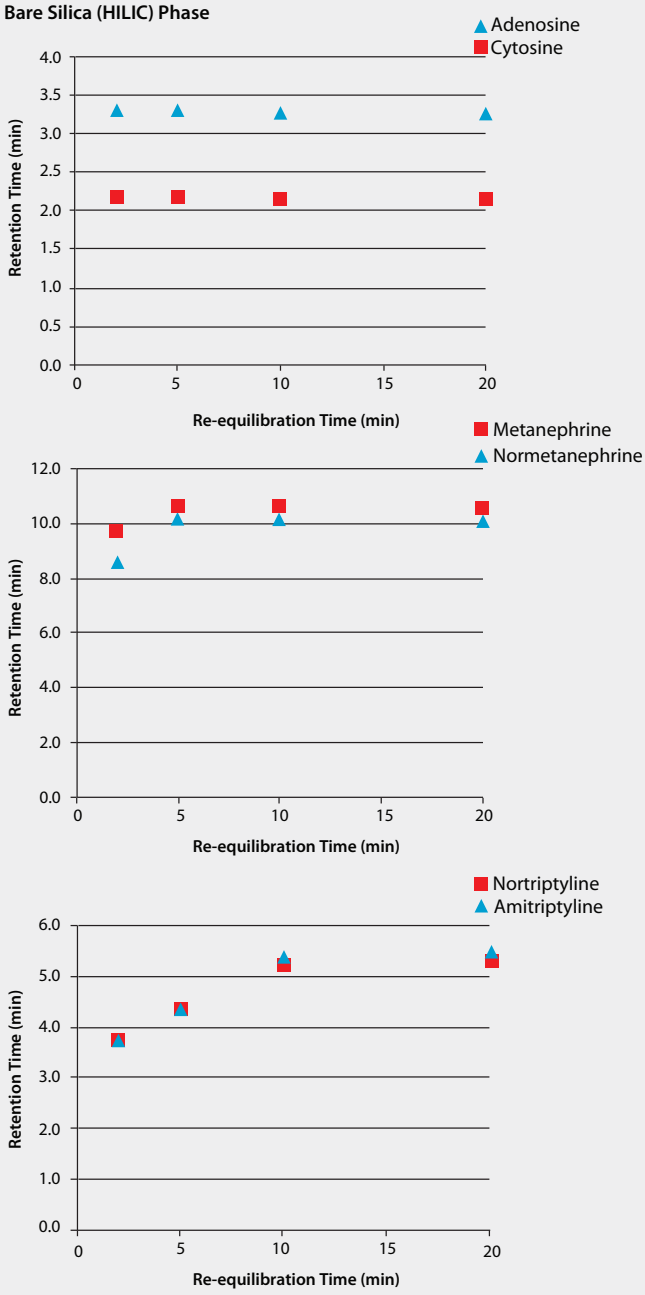



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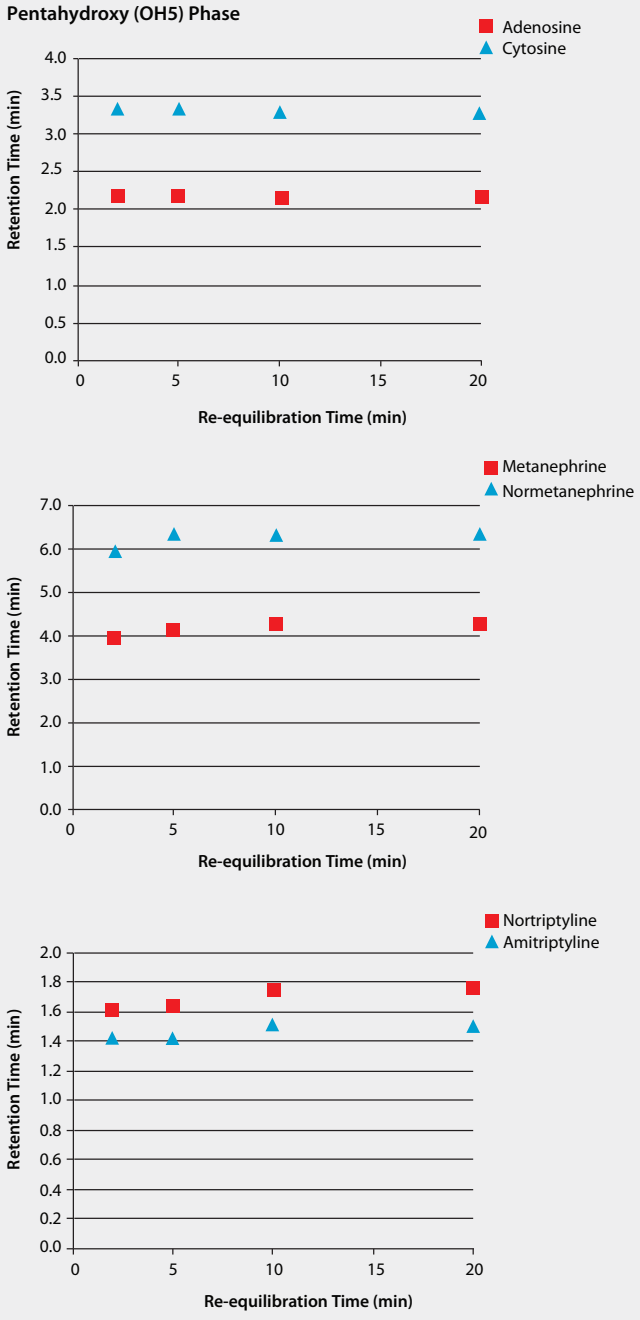
**Figure 7. Retention as a Function of Re-equilibration Time**




## Conclusions

HILIC gradient methods are often run to levels of the aqueous component that is well outside the normal HILIC percentages. An initial assumption regarding reproducibility of gradient HILIC methods was that running gradients past the 70% acetonitrile level would have significant impact on the overall reproducibility. The present study shows that running to even 50% aqueous had only a small impact on reproducibility. Interestingly, when re-equilibration times were held constant, reproducible retention, selectivity and peak shapes were observed. Marked changes were however observed upon extending re-equilibration times, especially for basic analytes on the bare silica stationary phase. Each of the experiments suggests that the presence of ion–exchange mechanisms results in slower overall equilibration. The use of bonded HILIC phases such as the OH5 may provide additional control of retention times when ion-exchange is present.

During the development of reversed-phase methods it is often assumed that if one allows the system to equilibrate for an extensive amount of time, the results would be the same as if the system were allowed to equilibrate for "just enough" time. The results of this study show that a gradient method in HILIC mode may appear equilibrated by virtue of reproducible retention times, however with extended re-equilibration time, may change. It is therefore important that gradient HILIC methods specify the re-equilibration times in order to ensure reproducible and reliable results.

### Featured Products

| Description | Cat. No. |
|---|---|
| Ascentis Express HPLC Columns | |
| HILIC, 10 cm x 3.0 mm I.D., 2.7 µm | 53970-U |
| OH5, 10 cm x 3.0 mm I.D., 2.7 µm | 53769-U |