TN-1050
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Impact of pH on the Purity and Yield for Preparative Separations

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Introduction

The introduction of new silica-based media in the last four years, that are stable to either acidic or basic conditions, provides the chemists more flexibility in choosing mobile phase conditions. Two of the newer media, Gemini® and Gemini®-NX, are available in analytical dimensions and are also packed in the larger preparative 21.2, 30 and 50 mm diameter dimensions using the Axia™ technology. This work compares and demonstrates the utility of these new media to improve throughput in the preparative and kilo laboratories.

Gradient conditions at low pH using volatile TFA have routinely been used for preparative separations due to the pH limitation of silica-based columns. The addition of Gemini and Gemini-NX in Axia-packed preparative column dimensions add new opportunities to achieve better purifications by operating at a pH range above 10. Gemini and Gemini-NX overcome pH limitations previously encountered with silica and hybrid particles.

Chemists always want to maximize throughput, but the two common problems encountered in preparative separations that limit throughput are low sample solubility and low resolution. This technical note compares the same preparative separations with low and high pH volatile buffers using Gemini and Gemini-NX media, and it highlights the advantages when operating at an elevated pH range even if the compounds have low solubility and/or low resolution.

Experimental

Gemini 5 µm C18 and Gemini-NX 5 µm C18 media, packed in 50 x 21.2 mm Axia columns, were used to investigate how low sample solubility and low resolution ultimately impact the yield and purity of the preparative purification. Gradient conditions were standardized using a 5 minute gradient from 5 % to 95 % ACN (Acetonitrile) with either a pH 2 mobile phase using 0.5 % TFA or a pH 10.5 mobile phase using 0.2 % NH_4OH (Ammonium Hydroxide). UV detection at 254 nm was used for all separations.

Three different sample mixtures of basic compounds with pK_a > 7 were separated using both high and low pH gradient conditions on the Gemini and Gemini-NX columns and the results were compared.

1. The diphenhydramine and propranolol sample mixture represents a difficult separation with low resolution. The two components are separated by only 0.1 minutes at low pH.
2. The lidocaine and diphenhydramine sample mixture represents a typical separation where the two components are better resolved by approximately 0.7 minutes using the low pH gradient conditions.
3. The diphenhydramine, oxybutynin and terfenadine sample mixture represents a situation where each compound must be isolated. The compounds are separated from each other by approximately 0.5 minutes at the low pH gradient conditions.

For each of these examples the initial sample load (represented by the bottom chromatogram in **blue** in each figure) is based on previous work under the low pH conditions where the resolution between the compounds was sufficient to isolate the first compound with purity greater than 95 % and a yield greater than 85 %. To represent the preparative separation problem of low analyte solubility, the same sample was loaded in a larger volume of DMSO in the second example (middle chromatogram in **red** in each figure). The third separation represents the situation where a chemist wants to increase productivity by increasing the mass loaded for each run (top chromatogram in **magenta** in each figure). The impact of further overloading the column is shown in these separations.

Results

In all experiments, the basic compounds have pK_a > 7 and when purified at low pH using 0.5 % TFA in the mobile phases the molecules are positively charged and more hydrophilic. With TFA in the mobile phase, the ionized bases have less hydrophobic interaction with the stationary phase as indicated by the shorter retention time and lower amount of acetonitrile required to elute the compounds. As seen in the chromatograms (**Figures 1A – 1B**, **2A – 2B**, and **3A – 3B**), diphenhydramine elutes with the low pH buffer at approximately 2.8 minutes when the acetonitrile concentration is at 50 %.

When separating the basic compounds at higher pH conditions, the compounds are more hydrophobic in their un-ionized state and are retained longer on the reversed phase columns. In all cases, a higher concentration of acetonitrile is required to elute these uncharged bases from the Gemini and Gemini-NX columns when using the pH 10.5 NH_4OH mobile phase (**Figures 4A – 4B**, **5A – 5B**, and **6A – 6B**). For example, diphenhydramine elutes with the high pH buffer at approximately 3.8 minutes when the mobile phase is 75 % acetonitrile, compared to 2.8 minutes with the low pH buffer when the acetonitrile concentration is 50 %.

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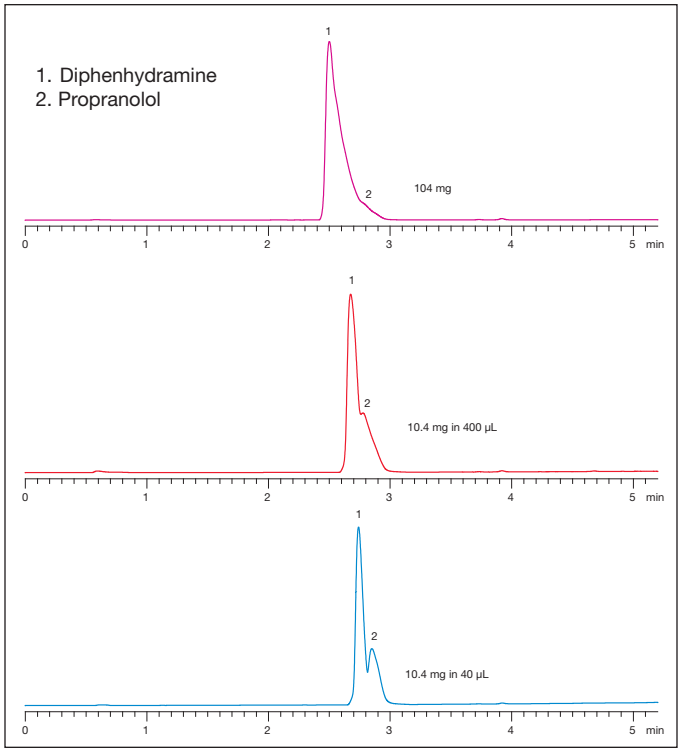
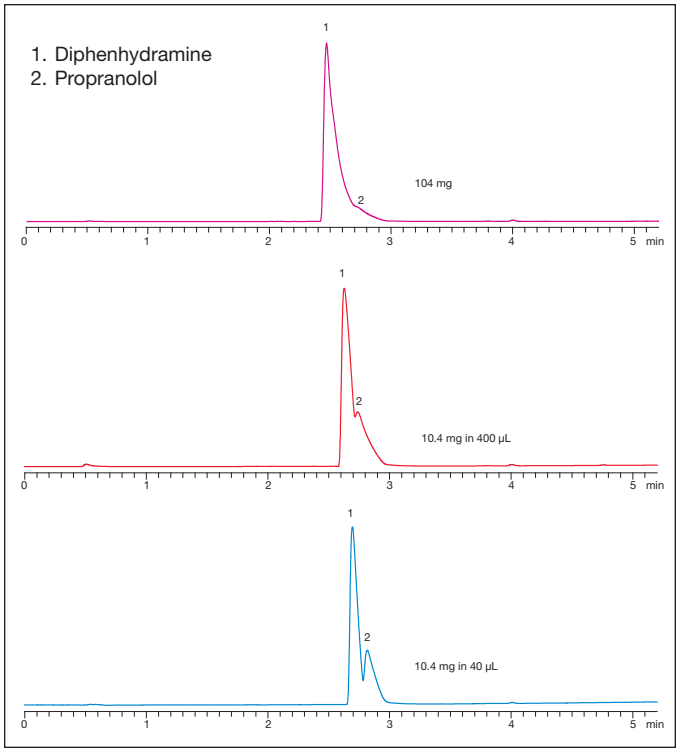
Results at low pH with 0.5 % TFA

Figure 1A
Gemini® Low pH with 0.5 % TFA



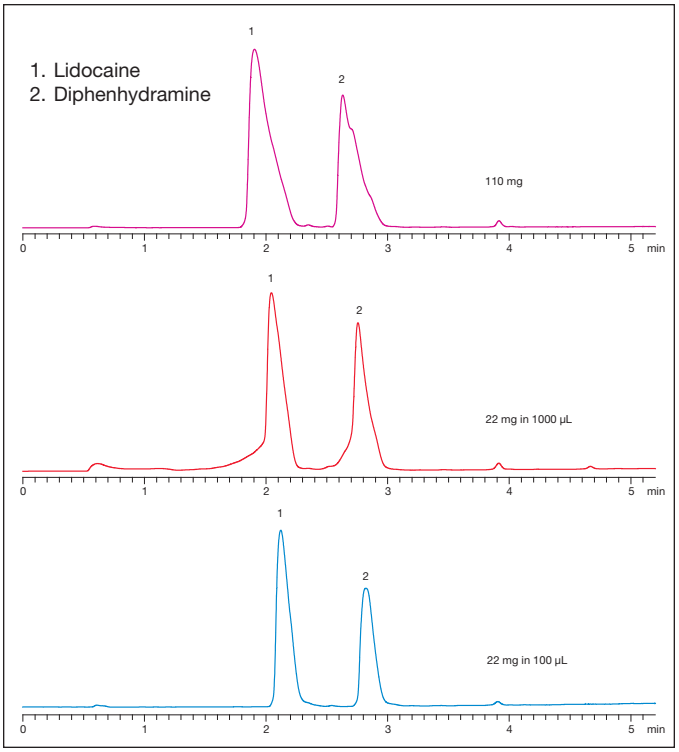
Diphenhydramine and propranolol are separated on the Gemini 5 µm C18 column. Diphenhydramine elutes at approximately 50 % acetonitrile when using the TFA gradient conditions. If a higher loading volume is used the resolution between the two compounds is lost, but the retention time for each of the peaks remains unchanged with this increased injection volume. If the sample mass is increased (same loading volume), the compounds elute earlier during the gradient indicating that the column is overloaded. With either the higher volume or the higher sample load there is no purification since the compounds merge together.

Figure 1B
Gemini®-NX Low pH with 0.5 % TFA



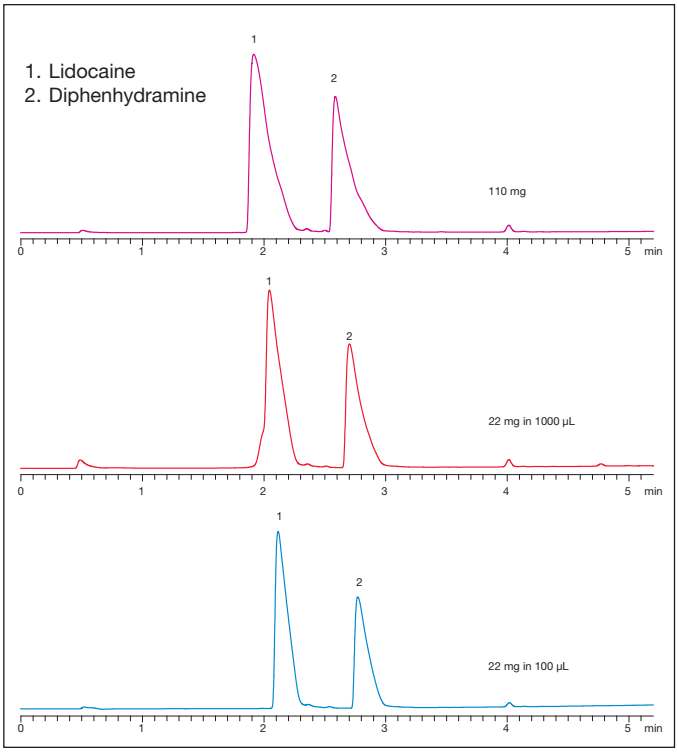
The separation of the two-component mixture is similar on Gemini-NX 5 µm C18 with a decrease in resolution observed when the higher volume of DMSO is used to load the sample. When the sample mass is increased to 104 mg the resolution between the compounds is lost since the two compounds elute so close together.

Figure 2A
Gemini® Low pH with 0.5 % TFA



Lidocaine is better resolved from diphenhydramine compared to the propranolol example in **Figure 1A** and higher sample loads (22 mg) are possible with this mixture. Lidocaine elutes from the column in approximately 40 % acetonitrile. When the loading volume is significantly increased, peaks begin to front due to the 1000 µL loading volume on the Gemini 5 µm C18 column and sample breakthrough occurs as indicated by the peak at the solvent front. The larger loading volume has a major impact on the peak shape for compounds with shorter retention times since they are eluting at much lower concentrations of acetonitrile. When the mass is increased 5X, the retention time decreases slightly and the compounds elute at slightly lower concentration of acetonitrile. With this higher load, the peak shape becomes broader for both compounds and the diphenhydramine begins to split.

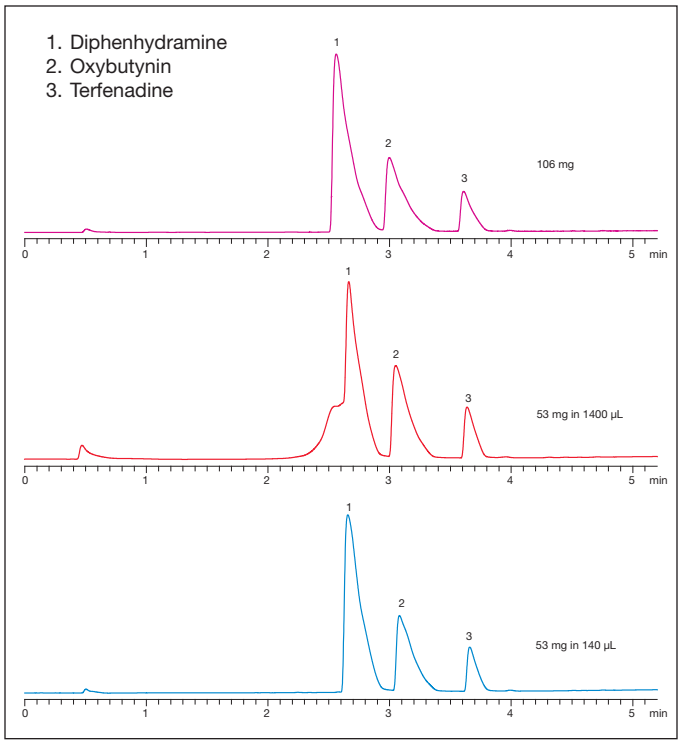
Figure 2B
Gemini®-NX Low pH with 0.5 % TFA



In this particular example there is very little difference between Gemini 5 µm C18 and Gemini-NX 5 µm C18 - the retention time and % acetonitrile required to elute lidocaine and diphenhydramine are very similar. The major differences observed are less peak fronting due to the higher volume of DMSO loading for Gemini-NX compared to Gemini and less peak splitting when the mass was increased to 110 mg.

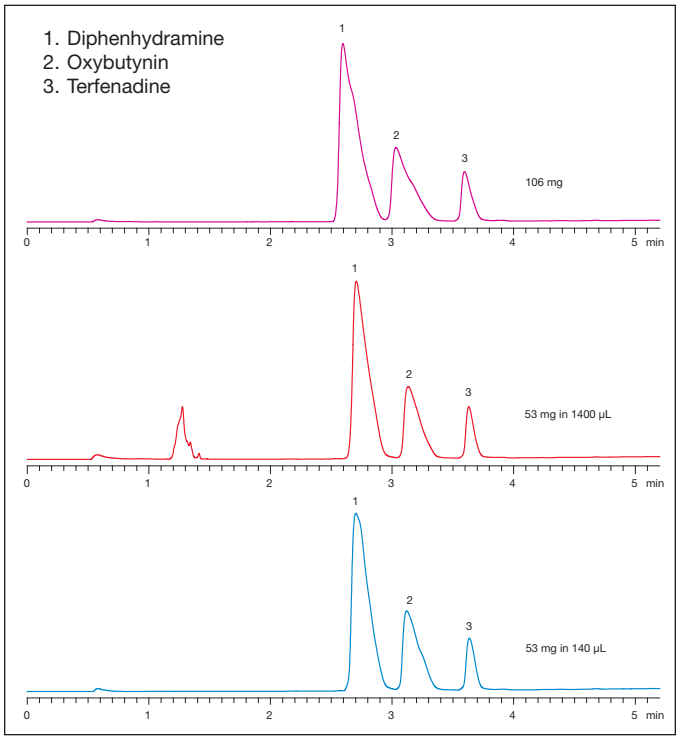
Results at low pH with 0.5 % TFA (continued)

Figure 3A
Gemini® Low pH with 0.5 % TFA



The three compounds are well resolved when using the Gemini media and a 53 mg load with the TFA buffer. When increasing the sample volume to 1400 µL the resolution is not lost but sample breakthrough at the solvent front is very evident. Increasing the mass load to 106 mg causes the compounds to elute slightly earlier but the resolution between diphenhydramine and oxybutynin is reduced and the peak shape begins to form the classic shark fin indicating that the column is severely overloaded.

Figure 3B
Gemini®-NX Low pH with 0.5 % TFA

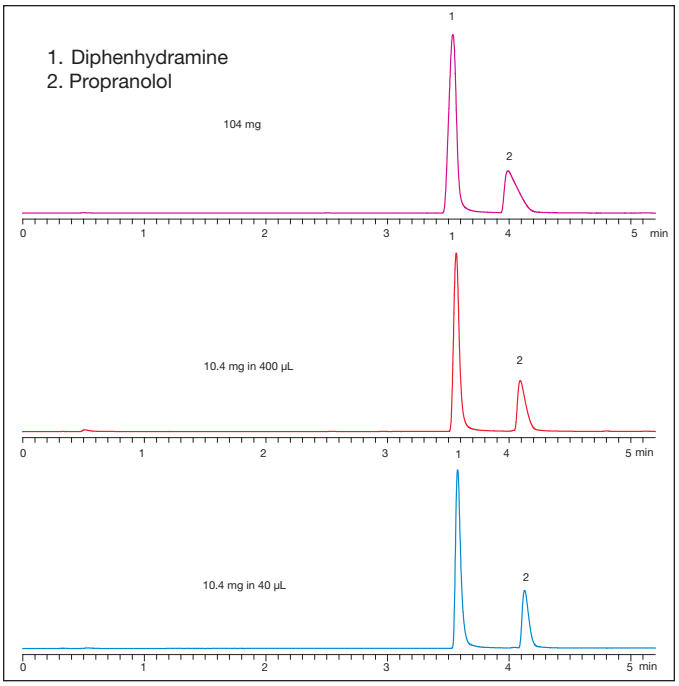


Gemini-NX 5 µm C18 also resolves the three component mixture at a 53 mg load. When the more dilute sample is loaded the 1400 µL injection deforms the peak shape due to the high volume of DMSO but there is less sample breakthrough at the solvent front compared to Gemini 5 µm C18. If the sample volume is limited to 700 µL the peak shape is maintained and there is no sample break through (data not shown). When the sample load is increased to 106 mg the compounds are still resolved, although they elute slightly earlier in the gradient, but there is no sample breakthrough at the solvent front.

Results at high pH 10.5 with 0.2 % NH_4OH

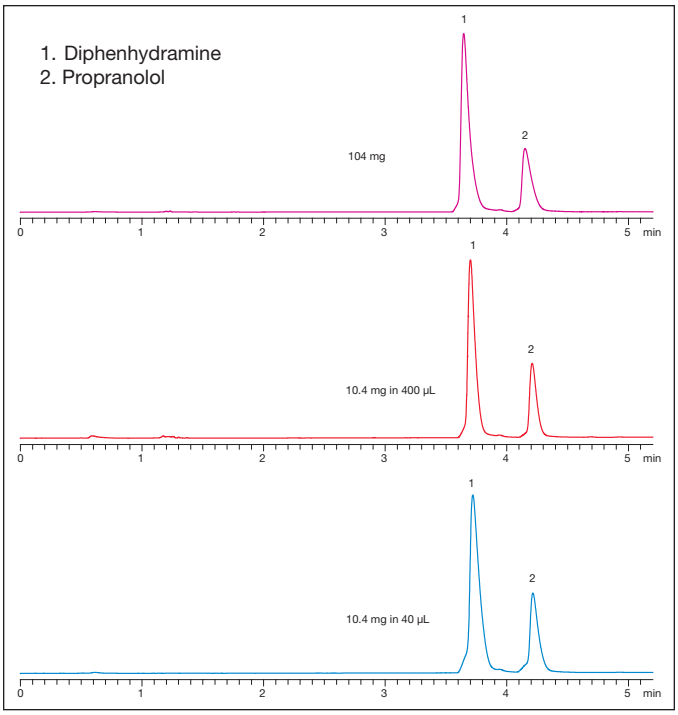
A dramatic change is seen when separating the basic compounds at pH 10.5 for both Gemini and Gemini-NX columns. At pH 10.5, the basic compounds are un-ionized and are more hydrophobic. The retention for the uncharged basic compounds increases and higher concentrations of acetonitrile are required to elute the compounds.

Figure 4A
Gemini® High pH 10.5 with 0.2 % NH_4OH



The resolution of diphenhydramine and propranolol is very much improved at the higher pH and the two compounds are now separated by 30 seconds instead of 6 seconds. The uncharged diphenhydramine requires 75 % acetonitrile to elute it from the column. Increasing the sample volume from 140 µL to 1400 µL does not affect the separation and increasing the sample load to 10X does not cause any loss of resolution.

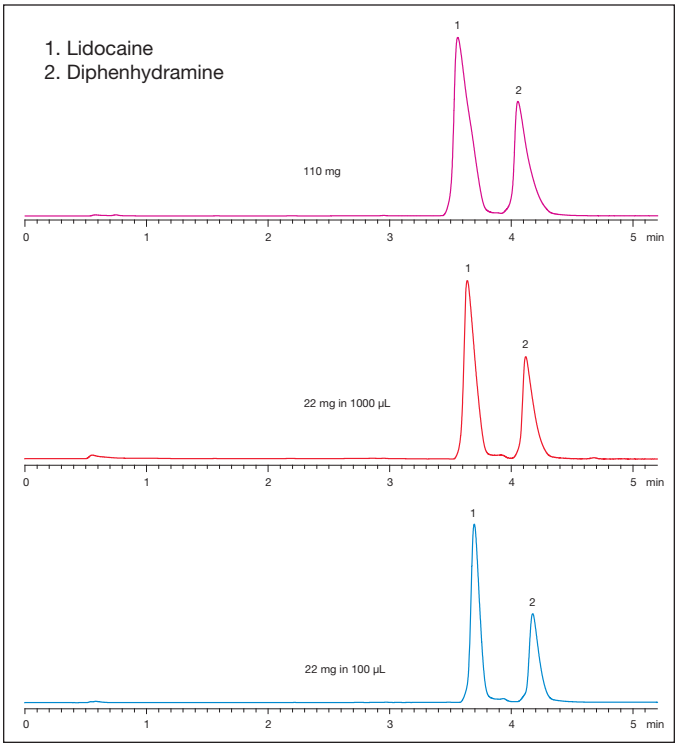
Figure 4B
Gemini®-NX High pH 10.5 with 0.2 % NH_4OH



Gemini and Gemini-NX provide similar resolution and separations for these mixtures. The retention time and acetonitrile required to elute the compounds increases when operating at pH 10.5. The higher loading volume and higher mass loading has less impact on the peak shape and retention and the peaks are less distorted when the purification is performed at pH 10.5.

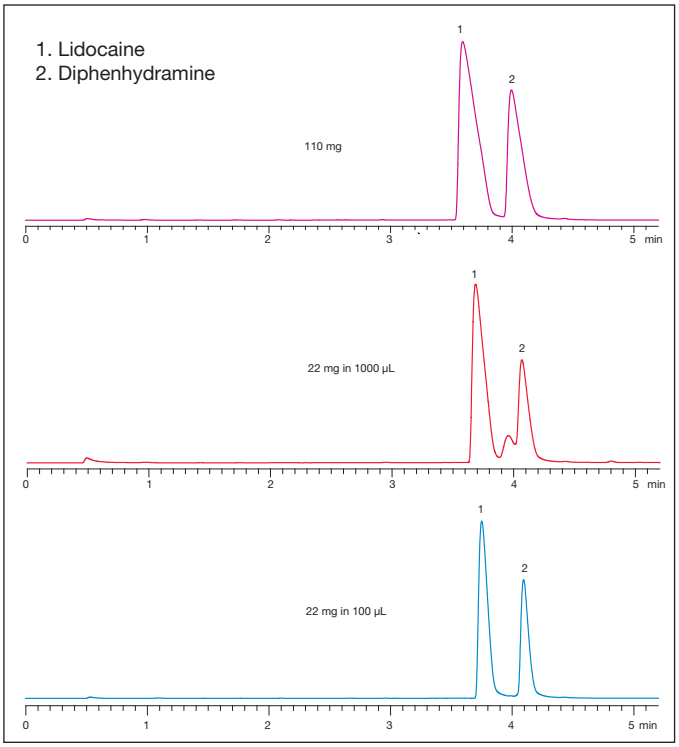
Results at high pH 10.5 with 0.2 % NH_4OH (continued)

Figure 5A
Gemini®-NX High pH 10.5 with 0.2 % NH_4OH



The preparative separation of diphenhydramine and lidocaine is also improved at the elevated pH 10.5 conditions compared to the low pH 2 conditions (compare **Figure 2A** and **5A**). With the higher pH mobile phases, the separation is not sensitive to the sample volume since the 1000 µL injection did not cause a distortion of the peak (red). Although increasing the sample mass resulted in a small increase in peak width, the resolution was still maintained and the retention time did not shift.

Figure 5B
Gemini®-NX High pH 10.5 with 0.2 % NH_4OH



With Gemini-NX the retention time and resolution for lidocaine and diphenhydramine are similar to Gemini and these compounds are also retained longer at pH 10.5 compared to pH 2. There is some peak splitting that occurs when the sample volume is increased to 1000 µL DMSO but the higher mass loading only causes a slight broadening of the peak and shorter elution time. The resolution between the peaks is not affected by the increased mass loading.

Figure 6A
Gemini® High pH 10.5 with 0.2 % NH_4OH

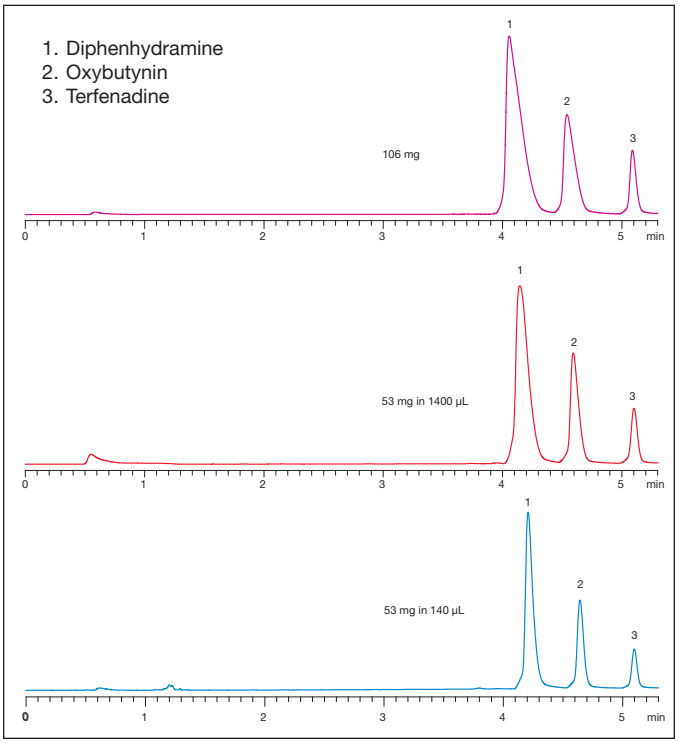
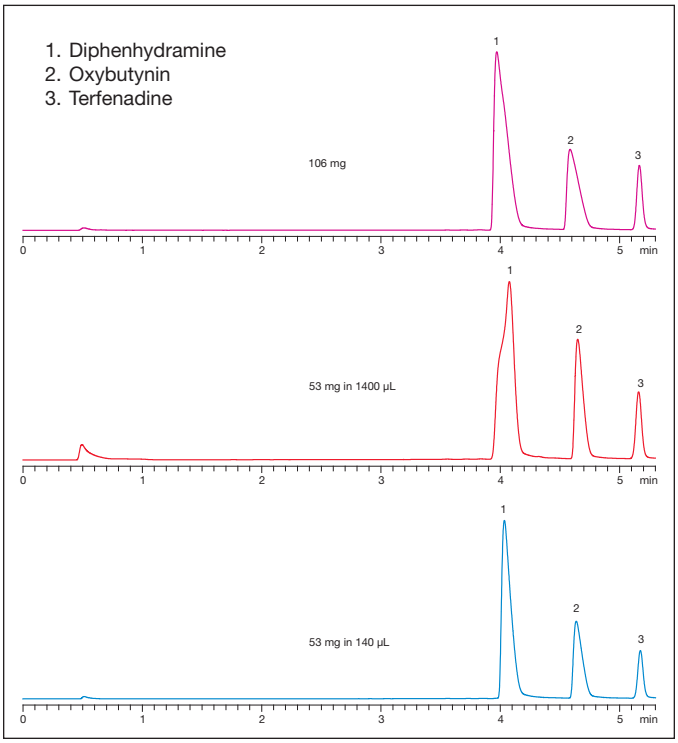


Figure 6B
Gemini®-NX High pH 10.5 with 0.2 % NH_4OH



The separation of the three-component mixture shows similar improvement in resolution and results at the higher pH condition. Increasing the loading volume or increasing the mass loading has less affect on the peak shape and retention when operating at pH 10.5 compared to operating at pH 2 (compare **Figure 3A** and **6A**). The sample mass could be increased further.

As seen with the previous two separations, these three compounds require a higher % acetonitrile to elute from the Gemini-NX column when the mobile phase is at pH 10. The resolution between the peaks is similar to Gemini and the resolution between the compounds is maintained as the sample volume and/or sample mass is increased. There is no loss of resolution and only a very slight shift in retention time as the sample mass increases indicating that much more sample could be loaded on the column.

Conclusions

For preparative separations of basic compounds the use of a high pH mobile phase is very advantageous:

1) At pH 10.5 the basic compounds are un-ionized and more hydrophobic, allowing increased interaction with the stationary phase.

2) Higher concentrations of acetonitrile are required to elute the compounds.

3) Separations are less affected by injection volume allowing more dilute samples to be purified.

4) Peak distortion due to column overloading is reduced and higher sample loads can be purified in a single run.

In all cases studied, the higher pH buffer allowed higher mass loading reducing the number of purification cycles required. The increased resolution at the higher pH also resulted in higher purity and yield for the desired compounds.

Operating at the higher pH range also provides an alternative choice for the chemist to use a smaller diameter column with the higher sample load. The smaller diameter column reduces the amount of solvent consumed and reduces the volume of the collected fractions.

Gemini-NX maintains similar retention characteristics, resolution, and capacity compared to the first generation Gemini product. Gemini-NX is a new standard media for high efficiency preparative separations utilizing a patent-pending organo-silica grafting process that incorporates high stabilizing ethane cross-linking into its structure. This technology provides resistance to high pH attack at elevated temperature, which is critical for preparative separations but also maintains the high efficiency and mechanical strength of a silica particle that allows the Axia™ process to be utilized to pack these preparative columns.

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Phenomenex products are available worldwide. For the distributor in your country, contact Phenomenex USA, International Department at international@phenomenex.com.

Ordering Information

Analytical		Part No.
Gemini-NX 5 µm C18	50 x 4.6 mm	00B-4454-E0
	150 x 4.6 mm	00F-4454-E0
	250 x 4.6 mm	00G-4454-E0
Gemini 5 µm C18	50 x 4.6 mm	00B-4435-E0
	150 x 4.6 mm	00F-4435-E0
	250 x 4.6 mm	00G-4435-E0

Axia-Packed Preparative		Part No.
Gemin-NX 5 µm C18	50 x 21.2 mm	00B-4454-P0-AX
	150 x 21.2 mm	00F-4454-P0-AX
	250 x 21.2 mm	00G-4454-P0-AX
Gemini 5 µm C18	50 x 21.2 mm	00B-4435-P0-AX
	150 x 21.2 mm	00F-4435-P0-AX
	250 x 21.2 mm	00G-4435-P0-AX

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