HPLC Method Development: Standard Practices and New Columns

Ed Kim Application Engineer March 16, 2010

What are Some Standard Method Development Practices?

- Follow preferred method development scheme and do "hands-on" method development – based on selectivity-changing parameters e.g., pH, column, or mobile phase types
- 2. Use method development software run a few predictive runs and get prediction for best method
- 3. Evaluate multiple columns/multiple mobile phases in a manual or automated fashion, determine best results (e.g., using valve switching to change between columns, software to track peaks etc.)

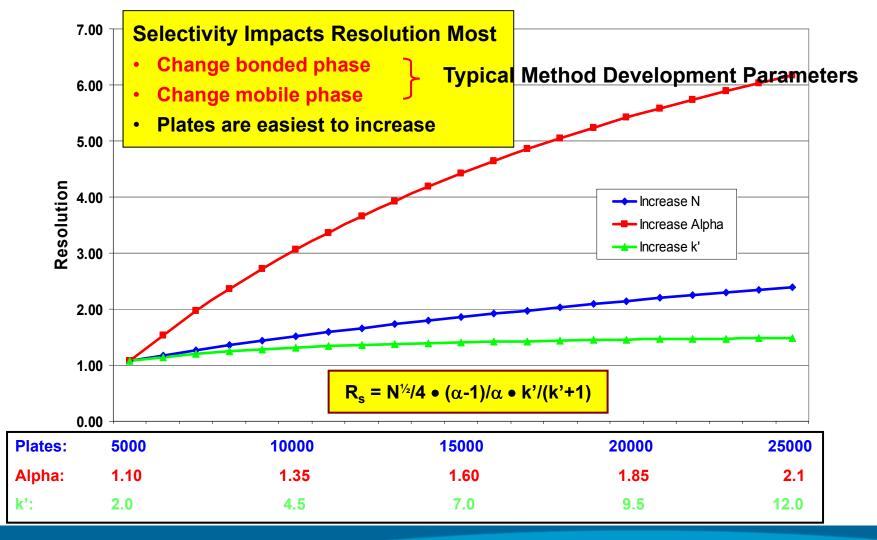
Practice #1 is used by many and incorporates "hands on" learning.

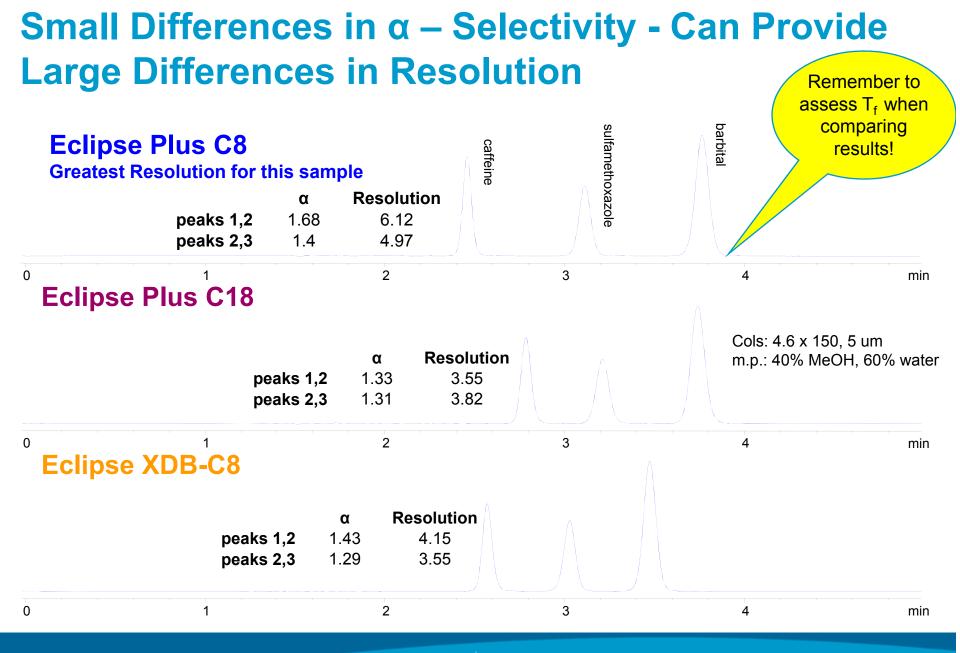
It requires nothing special to make it work, so we will work through a method development scheme for this practice.

What Makes a Good Starting Point for Hands-on Method Development?

- 1. Choose a C18 column many choices, well known technologies
 - a) A short column minimizes method development time
 - b) Smaller particle sizes provide the resolution needed in a short time
 - Newer C18 column choices can improve results based on efficiency, and improved peak shape
- 2. Choose a simple mobile phase reliable, works with many samples
 - a) Phosphate buffer pH 3, TFA, or formic acid in aqueous portion
 - b) Acetonitrile or methanol as organic modifier
- 3. Adjust mobile phase to get the desired retention and resolution
 - a) Adequate resolution of all peaks, $R_s \ge 2.0$ requires highest efficiency
 - b) Retention of first peak preferred to be at least k = 1
 - c) Analysis time below 20 minutes shorter
 - d) Newer column choices, short columns with small particle sizes can provide more efficiency and resolution in a very short time, thereby speeding up method development

Typical Method Development Parameters - Effects of Selectivity, Efficiency, and Retention on Resolution





What Do We Want to Base Our Complete Method Development Scheme On? (Beyond the Start)

Changing selectivity to improve resolution with the two key parameters – mobile phase and bonded phase

Mobile phase (1st choice to change because it's easy)

- Mobile phase organic modifier (ACN, MeOH, etc.)
- Mobile phase pH over a wide pH range pH 1-12 if needed

Bonded phase (provides a lot of optimization potential)

- Many choices other than C18 C8, Phenyl, CN (most orthogonal!)
- AQ for highly aqueous mobile phases
- Choose new columns with best peak shape performance

New Column Choices To Use for Standard Method Development

1. Shorter columns with smaller particle sizes

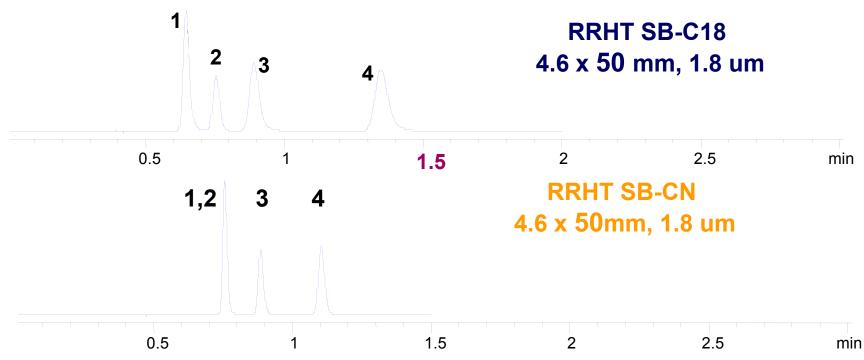
- Rapid Resolution High Throughput Columns (RRHT) with a 1.8 μm
 particle size 50 mm or shorter for fastest method development
- Longer columns for maximizing efficiency
- Many bonded phase choices for a complete method development scheme

2. Improved bonded phases for superior performance

- Better peak shape and efficiency result in more resolution
- Critical to the best methods
- New Eclipse Plus columns can deliver improved performance

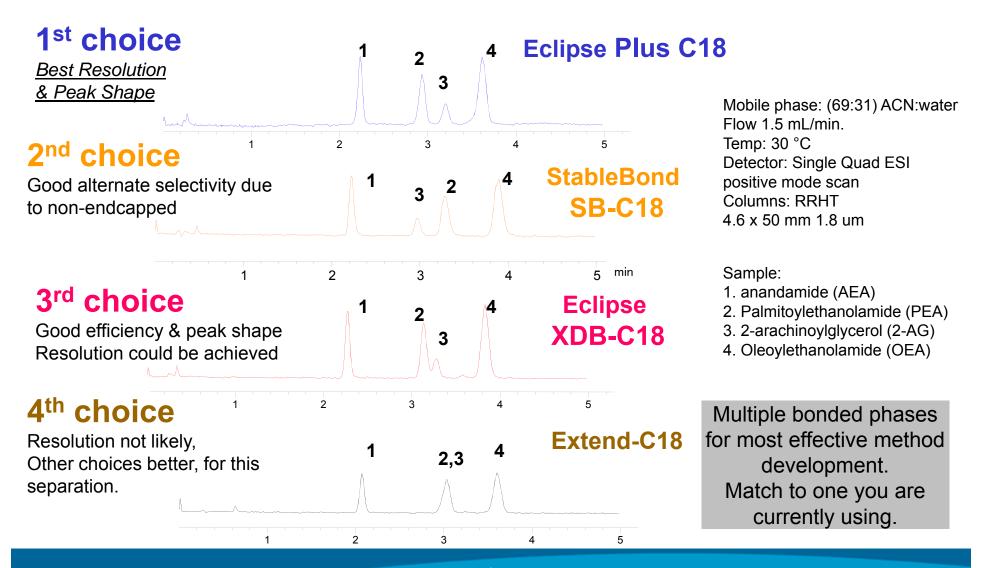
1. Start with Short RRHT C18 Columns with Highest Efficiency for Fastest Method Development

Column: 4.6 x 50mm, 1.8um Mobile Phase: A= 0.1% Formic Acid, B=ACN + 0.1% Formic Acid (95:5) Flow rate: 1.5 mL/min Inj. Vol: 2 ul Sample: Xanthines: 1. 1-methylxanthine, 2. 1,3-dimethyluric acid, 3. 3,7-dimethylxanthine, 4. 1,7-dimethylxanthine

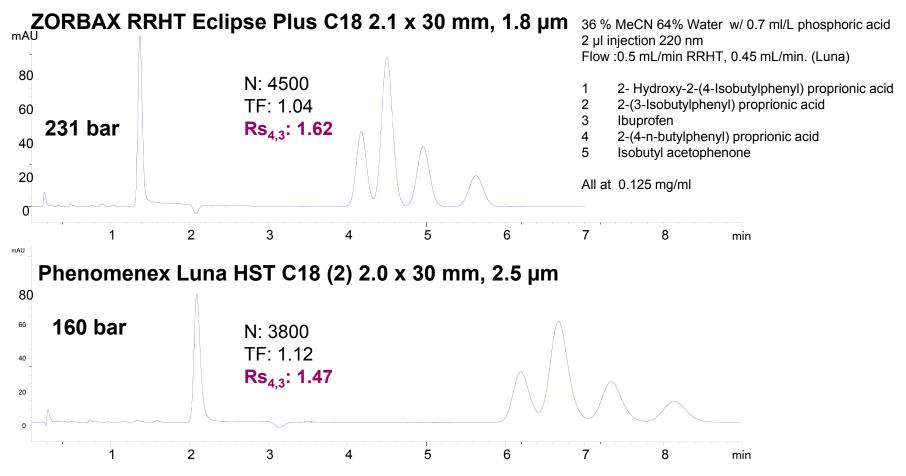


- ➤ The C18 gives the most effective resolution and with a 4.6 x 50 mm, 1.8um column the initial results are achieved in just a couple of minutes, with the highest efficiency.
- ➤ The 50 mm length is short and fast methods can be approximated or completed in just minutes!!

1. Start Method Development with RRHT Columns: Different ZORBAX RRHT C18 Bonded Phases for Max Selectivity



2. Agilent Rapid Resolution HT Eclipse Plus C18 Provides Superior Peak Shape and Efficiency

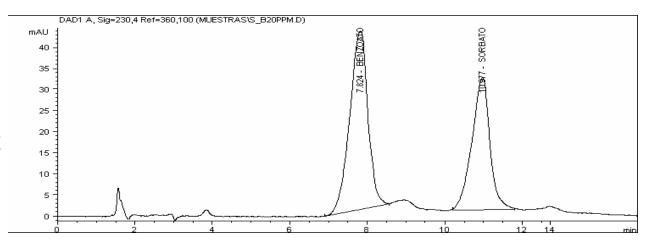


ZORBAX RRHT has at least 18% more efficiency than Luna HST C18(2) That means greater than baseline resolution when needed.

2. Choose New Eclipse Plus C18 for Superior Efficiency and Peak Shape

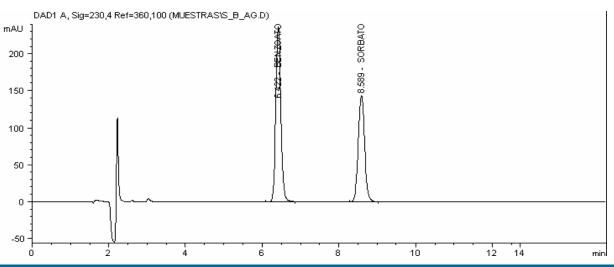
Analysis on Other C18

- Poor sensitivity
- ➤ Due to broad peaks
- ➤ Peaks do not tail, but are distorted.



Analysis on Eclipse Plus

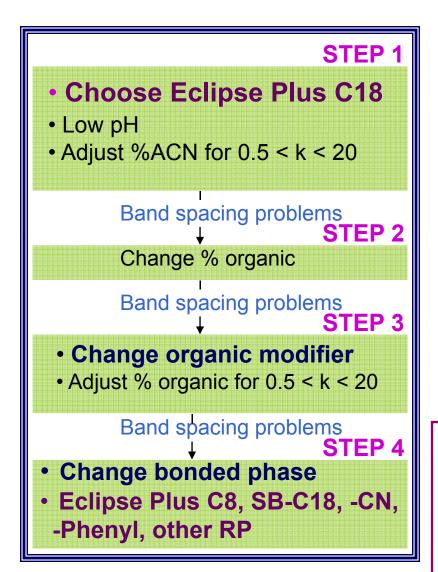
- ➤ Better Sensitivity
- ➤ Sharp, Efficient Peaks
- ➤ Excellent peak shape



How Do We Implement These New Column Choices in the Method Development Process?

- □ Follow a method development process around standard practices ONLY when the first steps allow you to incorporate new products for fast, good results!
- ☐ A method development scheme based on simple strategies for optimizing selectivity and resolution can incorporate new products.
- ☐ The 3 key parameters must be:
 - mobile phase
 - mobile phase pH
 - bonded phase

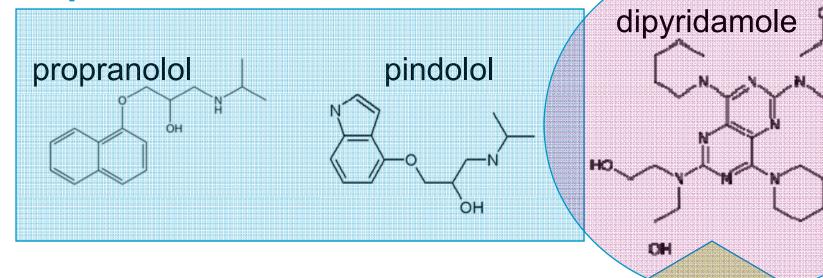
First Steps in a Method Development Scheme



- ➢ Select a high quality C18 or C8 bonded phase first for good peak shape, retention and resolution with typical acidic, basic and neutral samples.
- ➤ Optimize the organic component of the mobile phase to change selectivity
- Choose alternate bonded phases to completely optimize method, if needed
- Choose SB-C18 for pH 1-2
- ➤ With new RRHT column choices, these steps can be done quickly, making it possible to truly find the best method.

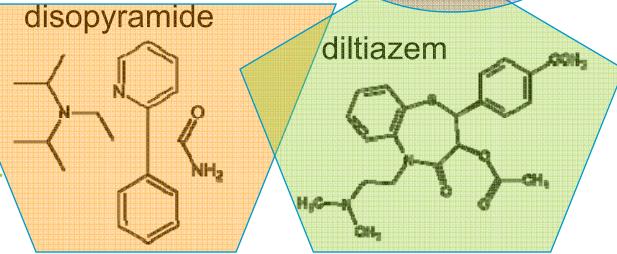
Let's Look at Method Development of These

Compounds

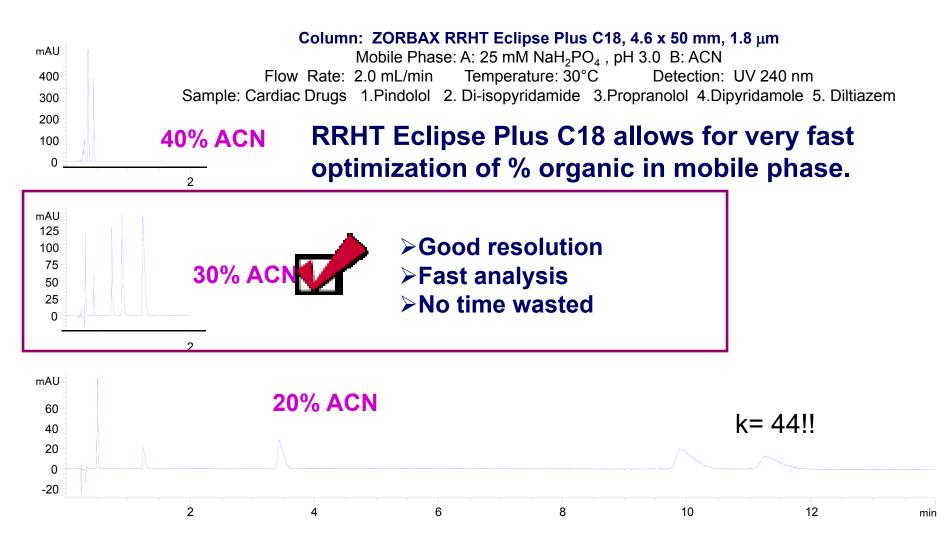


β-blocker
Anti-arrhythmic
Vasodilator

Ca⁺² channel blocker



Start at Low pH, Adjust % Organic Eclipse Plus C18 Cardiac Drugs with Acetonitrile

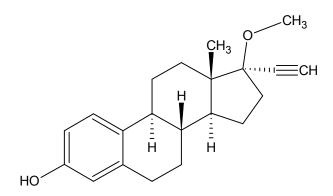


Steroidal Compounds

estriol

estradiol

ethinyl estradiol

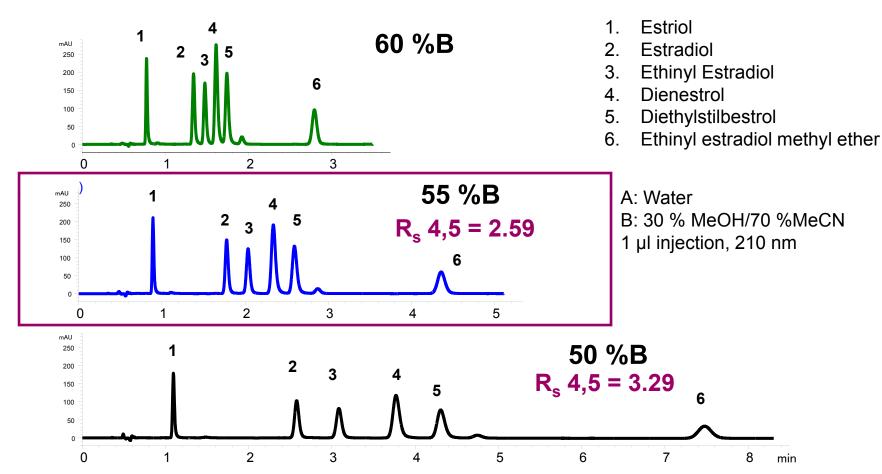


dienestrol

diethylstilbestrol

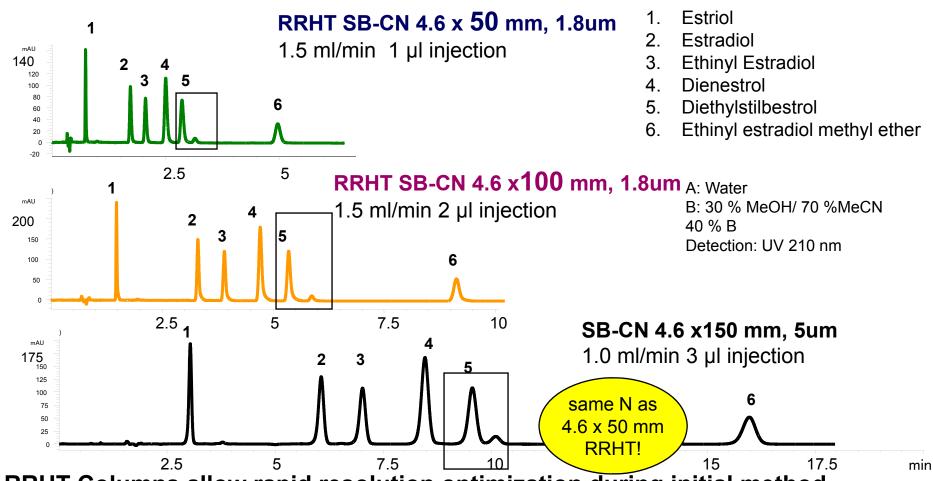
ethinyl estradiol 3-methyl ether

Mobile Phase Optimization on Short RRHT 4.6 x 50 mm, SB-CN Column



> RRHT columns allow for very fast optimization of % organic in mobile phase.

Comparison of Optimal Conditions On Columns with Different Dimensions – Length, Particle Size, Resolution

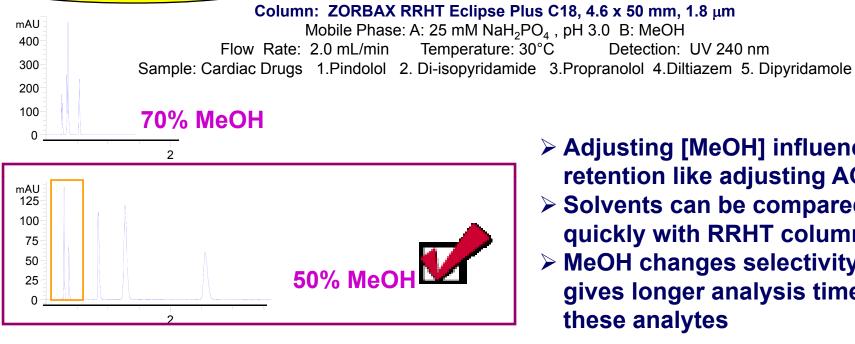


RRHT Columns allow rapid resolution optimization during initial method development. Column lengths compared quickly.

Why Do We Recommend Changing Organic Modifier Before Changing the Bonded Phase?

- ▶It's easy acetonitrile and methanol are readily available!
- ➤ It works on any bonded phase, so it gives you a tool to possibly optimize a separation no matter the column choice!

Start at Low pH on Eclipse Plus C18 RRHT Cardiac Drugs with Methanol, Changing Organic Modifier

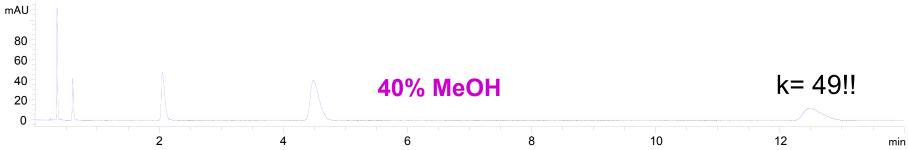


Remember to use buffer!

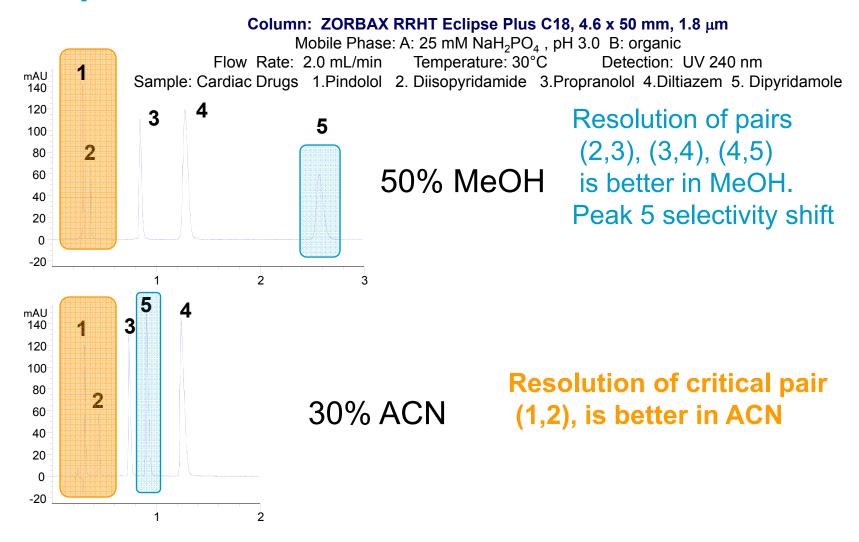
- Adjusting [MeOH] influences retention like adjusting ACN
- Solvents can be compared quickly with RRHT columns

Detection: UV 240 nm

MeOH changes selectivity and gives longer analysis time for these analytes

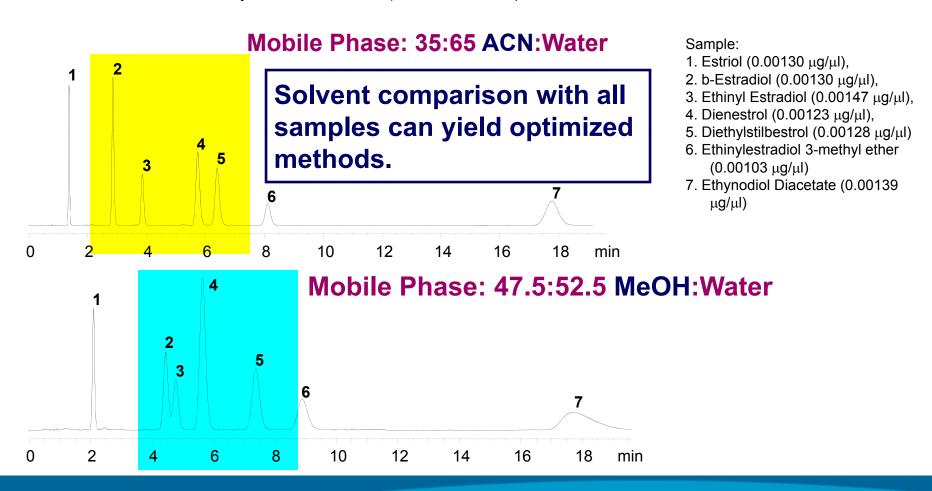


Method Development - Change Organic Modifier Comparison of Acetonitrile and MeOH



Change Mobile Phase - Acetonitrile vs. Methanol on ZORBAX Eclipse XDB-CN with Estrogens

Column: ZORBAX Eclipse XDB-CN Column Dimensions: 4.6 x 150 mm, 5 μ m Mobile Phase: As shown Flow Rate: 2.0 ml/min Injection Volume: 2.00 μ l Column Temperature: 25 °C Detector: UV, 210 nm



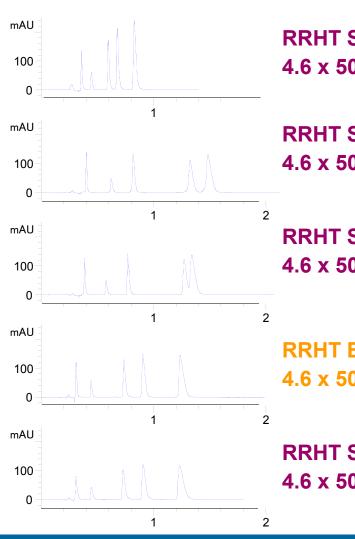
Why Is Changing the Bonded Phase Effective?

- ▶Differences in interactions between polar and non-polar compounds.
- ➤Other types of interactions with a bonded phase can be exploited (pi-pi interactions etc.)
- These all change with bonded phase!
- ➤ Changing the bonded phase can improve selectivity/ resolution, reduce analysis time

When you use RRHT (1.8um) columns, the comparison of bonded phases can be done quickly!!

➤ You must have multiple RRHT column choices available

Bonded Phase Selectivity Differences in 30% ACN



RRHT SB-CN 4.6 x 50 mm, 1.8 μm

RRHT SB-Phenyl 4.6 x 50 mm, 1.8 μm

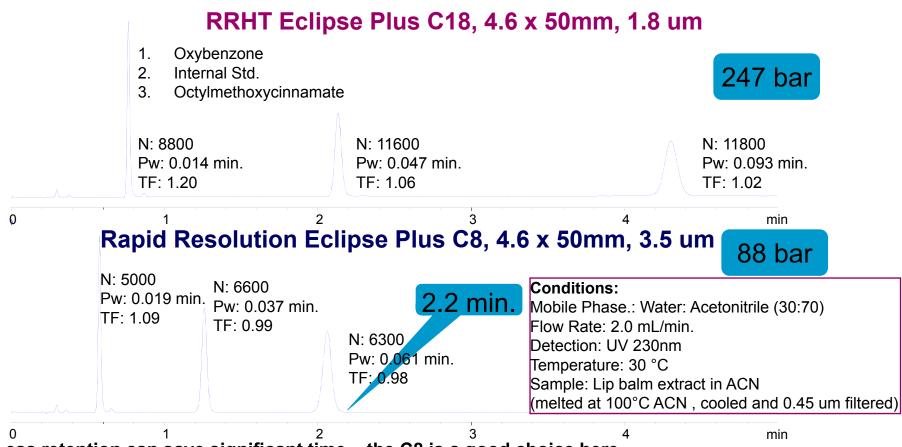
RRHT SB-Aq 4.6 x 50 mm, 1.8 μm

RRHT Eclipse Plus C18 4.6 x 50 mm, 1.8 μm

RRHT SB-C18 4.6 x 50 mm, 1.8 μm

- 5 Different bonded phases compared
- Analysis time of each run is only 2 minutes
- Comparison among phases done in optimum % organic for SB-CN
- ➤ The fast runs mean a comparison can be done even if you have a good separation on the C18
- More chances to optimize!

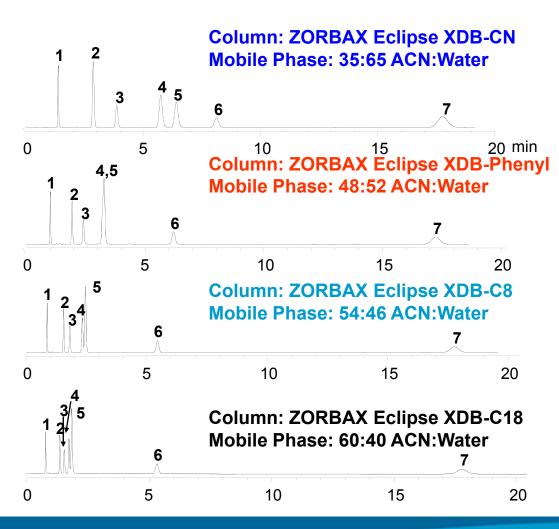
Selectivity/Bonded Phase Choices: Eclipse Plus C18 vs. C8 and RRHT vs. RR



Less retention can save significant time – the C8 is a good choice here.

The RRHT column is delivering the efficiency and resolution expected, but the C8 bonded phase may be the better choice.

Selectivity of Polar Phases Provides Optimum Separation of Steroids Versus Non-Polar C18/C8



Column Dimensions: 4.6 x 150 mm, 5 μm

Flow Rate: 2.0 ml/min Injection Volume: 2.00 µl Column Temperature: 25 °C

Detector: UV, 210 nm

Sample:

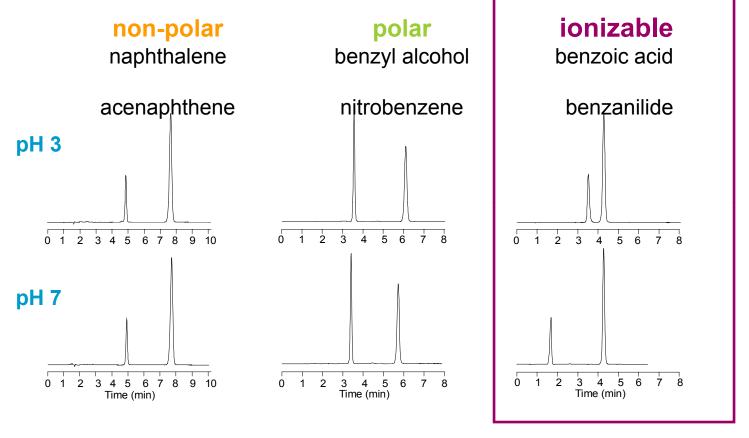
- 1. Estriol (0.00130 μg/μl),
- 2. b-Estradiol (0.00130 μg/μl),
- 3. Ethinyl Estradiol (0.00147 µg/µl),
- 4. Dienestrol (0.00123 μg/μl),
- 5. Diethylstilbestrol (0.00128 μg/μl)
- 6. Ethinyl estradiol 3-methyl ether (0.00103 μ g/ μ l)
- 7. Ethynodiol Diacetate (0.00139 µg/µl)

➤ Eclipse XDB-CN is the optimum bonded phase – the only one that can resolve all the components in under 20 minutes.

So then Why is pH Critical to Method Development?

- >What compounds are pH sensitive?
- ➤ How does pH affect retention and resolution?
- >How does pH influence method development?
- >How does pH influence column choice?

When Does pH affect Resolution? Compound Type Comparison



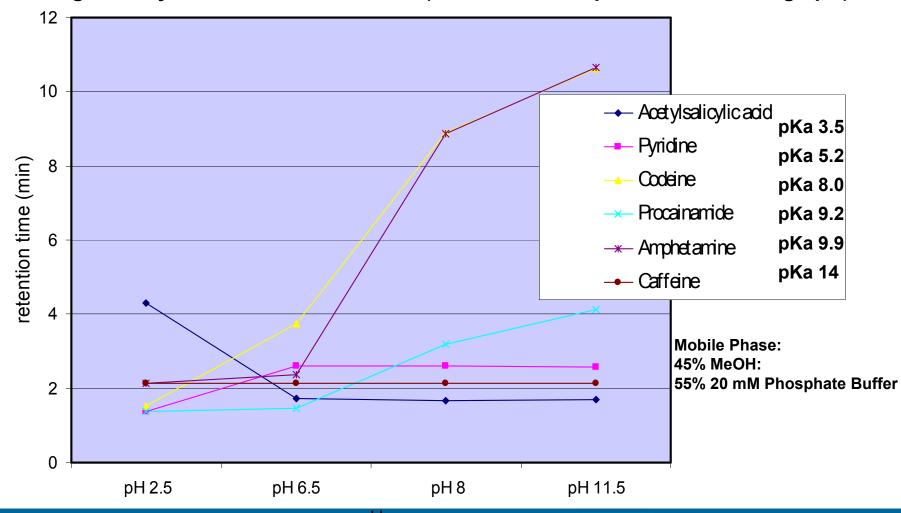
 lonizable compounds – acids and bases can change retention and selectivity the most with changes in pH

Change in Retention with pH for Ionizable Compounds is Key to Method Development

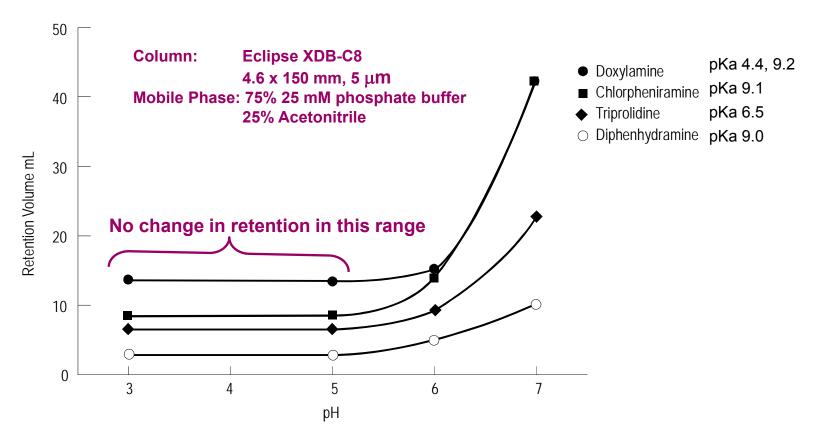
- ➤ Non-charged analytes have better retention (i.e. acids at low pH and bases at high pH)
- ➤ Silanols on silica ionize at mid-pH, increasing retention of basic analytes
- (i.e possible ion-exchange interactions)
- Choose mobile phase pH to optimize retention and selectivity during method development
- ➤ Eclipse Plus can be used over a wide pH range
- Other choices exist for high pH

Change in Retention with pH for Ionizable Compounds is Compound Dependent

Non-charged analytes have better retention (i.e. acids at low pH and bases at high pH)



Retention vs. pH for Basic Antihistamines - Less Variation in Retention at Low pH



- Slight changes in pH may dramatically change retention and selectivity
- Always evaluate retention changes with pH during method development

Therefore pH Should be Considered a Key Method Development Parameter

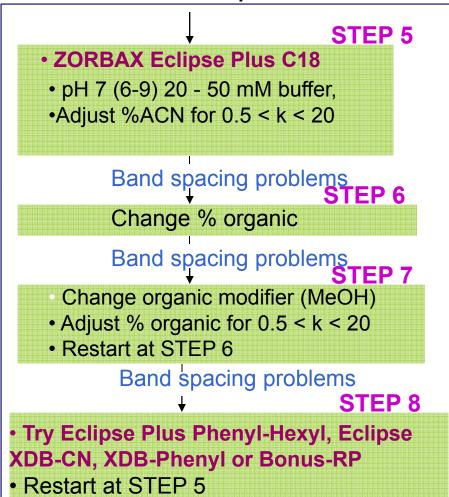
A single column can be used over a wide pH range making selectivity comparisons quick and reliable when pH is changed.

May have better selectivity in the pH range 3 - 8.

Other bonded phases can be reconsidered for mid pH, just like at low pH

Method Development Scheme – Adding Mid pH

From low pH



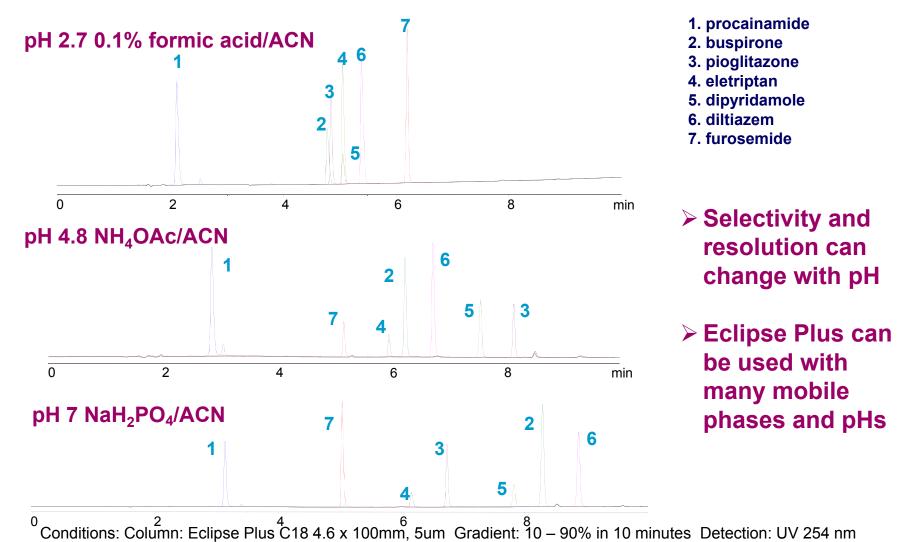
- > Mid pH can provide better selectivity
- ➤ It may be more compatible with your sample
- The process for investigating mid pH is the same as for low pH
- Eclipse Plus delivers outstanding performance at mid pH
- Alternate bonded phases should also be considered if improved selectivity is desired

Eclipse Plus Columns Are Ideal for Method Development at Low and Mid pH

- **Can be used from pH 2-9** → Can be used from pH 2-9
- Accommodates method development in low and mid pH ranges
- >Allows pH sensitive separations to be optimized

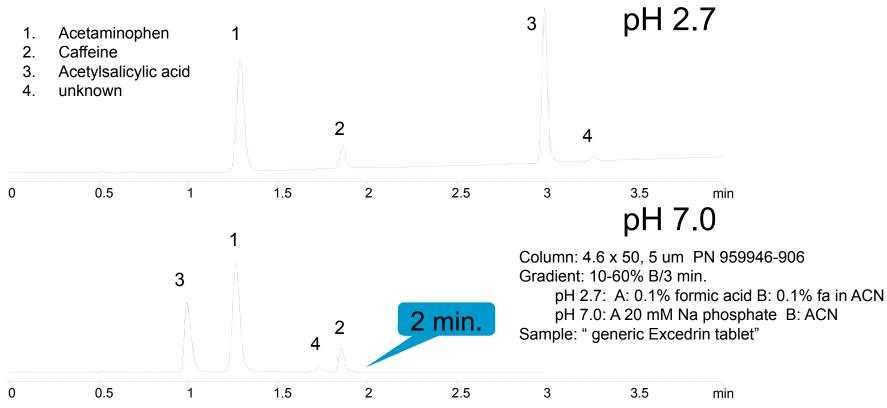
Excellent peak shape and efficiency achieved with new Eclipse Plus material

Eclipse Plus Can Be Used Over a Wide pH Range (pH 2 – 9) to Optimize Selectivity



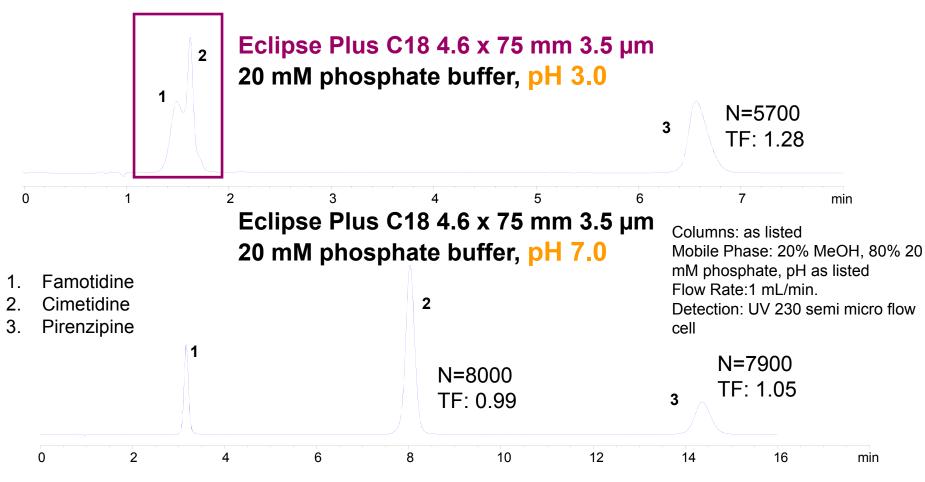
Selectivity Differences at pH 2 and pH 7 Can Be Dramatic





- ➤ Selectivity can change dramatically from pH 2 7 with many samples, such as for the ingredients in this analgesic tablet.
- > Eclipse Plus can be used under both conditions

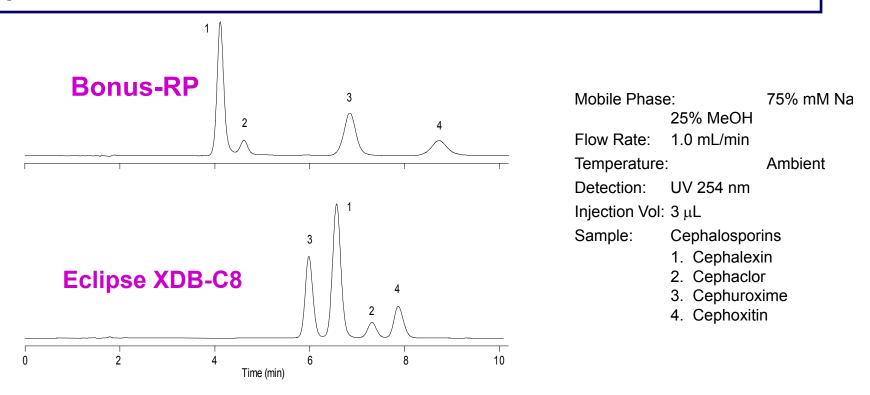
Selectivity Differences at Mid pH Key to Resolution Eclipse Plus Performs well at Low and Mid pH



- > For this sample resolution is not possible at pH 3, but is at pH 7
- > Eclipse Plus was used to experiment at both pHs

Try an Alternate Selectivity at Mid pH if Needed

- ➤ One choice for alternate selectivity is Bonus-RP.
- ➤ The alkyl-amide bonded phase provides very different selectivity at mid-pH



Mid pH was the second pH region to consider. High pH can also be considered if low or mid pH do not resolve all analytes.

Method Development at High pH – a Third Choice

From Mid pH

STEP 9

- ZORBAX Extend-C18
- pH 10.5 (9-12) 5 mM ammonia, or TEA,
 or 10 50 mM organic or borate buffers
- T = 25° C (ambient 40° C)
- Adjust MeOH for 0.5 < k < 20

Band spacing problems
STEP 10

- Change organic modifier (ACN or THF)
- Adjust for 0.5 < k < 20

Try different HPLC mode

Reasons to Consider High pH

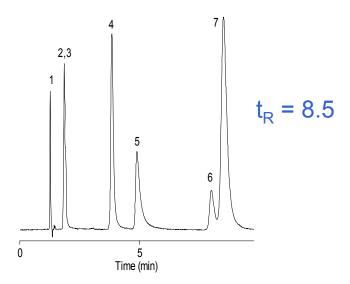
- Increase retention of basic compounds by analyzing them in non-charged form
- > Improve selectivity

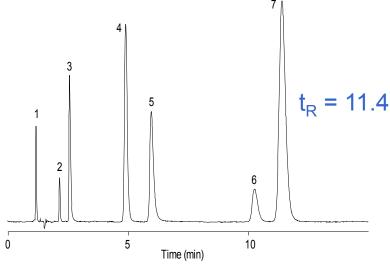
High pH Increases Retention of Antihistamines Extend-C18

Column: ZORBAX Extend-C18, 4.6 x 150 mm, 5 μ m Mobile Phase: See Below Flow Rate: 1.0 mL/min Temperature: RT Detection: UV 254 nm Sample: 1. Maleate 2. Scopolamine 3. Pseudoephedrine 4. Doxylamine 5. Chlorpheniramine 6. Triprolidine 7. Diphenhydramine

pH 7 30% 20 mM Na₂HPO₄ 70% MeOH







> The retention of the basic compounds in this sample increases at high pH.

Conclusions

New column choices, like 1.8um RRHT columns and Eclipse Plus bonded phases can enhance typical approaches to method development.

These columns provide fast analysis, allowing more options to be considered quickly, and with superior performance, the best results are readily achievable.

Since many compounds are ionizable, and retention and selectivity of these compounds are greatly impacted by pH, a method development scheme for LC methods should encourage varying pH.

Any method development scheme should also include the use of different bonded phases. Different bonded phases and columns can be selected by pH as well.

The third key aspect of method development is mobile phase and organic modifier and this is an effective tool for many separations.

Agilent Technical Support

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Select opt. 3, opt. 3, then option 1 for GC or option 2 for LC.

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