DEVELOPMENT OF A HIGHLY SELECTIVE, "PHENYL-MODIFIED" C18 BONDED PHASE FOR HPLC

Carl L. Zimmerman, Thomas J. Waeghe, Robert T. Moody, MAC-MOD Analytical, Inc., Chadds Ford, PA

In recent years, the use of highly purified, ultra-inert silicas as the base materials for high performance liquid chromatography columns has led to columns of unparalleled performance, stability and reproducibility. Excellent peak shapes can now be obtained for previously troublesome basic compounds. The C18 (ODS) phase is still the most popular bonded phase used in HPLC. However, for some compounds there is little difference in selectivity between C18 columns, even from different manufacturers.

When one is faced with poor selectivity on different C18 columns, it is common practice to investigate non-aliphatic bonded phases, in particular, those containing phenyl groups. The strong $\pi - \pi$ interactions between the bonded phase and the analytes often give improved selectivity for troublesome critical pairs. Unfortunately, many phenyl phases, even those with longer aliphatic chain linkers (for example, phenylhexyl), frequently offer insufficient hydrophobic retention, one of the main benefits of a C18 phase.

In this poster we will describe the development and commercialization of a new, highly stable, proprietary phase, ACE® C18-AR, which combines the retention characteristics of a classical C18 phase with the highly selective $\pi - \pi$ interactions of a phenyl phase. In addition to enhanced selectivity over classical C18 phases, ACE C18-AR possesses sufficient increased polarity to be compatible with pure aqueous mobile phases. A variety of examples will be presented to demonstrate the outstanding selectivity, stability and reproducibility of the new ACE C18-AR columns.

Introduction

C18 or ODS bonded phases were one of the first commercially available reversed phase columns. To this day, they remain the most popular reversed phase columns and are used in the majority of HPLC separations. The popularity of C18 is in part due to its historical use, but it also has many characteristics desirable for RP-HPLC including:

- Excellent stability. C18 columns have good stability over the pH range 2 – 9.
- Inertness. Due to the high coverage of the silica surface, C18 phases give the best peak shapes for basic compounds.
- Retention. C18 columns have excellent retention characteristics for hydrophobic compounds.

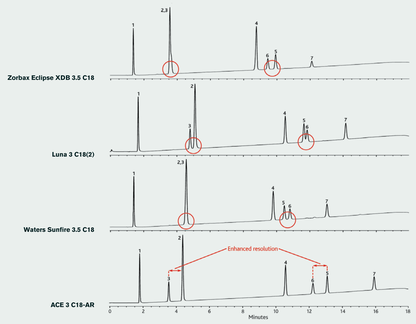
Due to its high hydrophobicity, however, a C18 phase has limitations that can impact the analysis of polar compounds such as those analyzed in the pharmaceutical industry. Some problems encountered with C18 are:

- Poor retention of very polar compounds
- Incompatibility with highly or totally aqueous mobile phases
- C18 lacks a selectivity "handle" and often fails to resolve similar polar compounds

ACE® C18-AR incorporates a unique, proprietary "phenyl modified" C18 chain which preserves all of the desirable characteristics of a classic C18 bonded phase while providing sufficient polar character to correct many C18 shortcomings. ACE® C18-AR columns provide:

- Hydrophobic retention similar to classic C18 phases
- Excellent peak shape for basic compounds
- Exceptional stability from pH 2 –9
- Improved retention of highly polar compounds
- Compatibility with 100% aqueous mobile phases
- Vastly improved selectivity for the separation of polar compounds

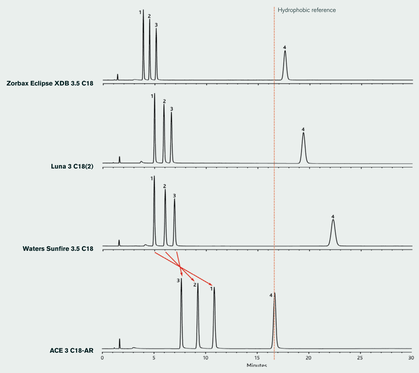
FIGURE 1: **Water Soluble Vitamins**



Sample: 1. pyridoxine 2. p-aminobenzoic acid 3. pantothenic acid 4. folic acid 5. cyanocobalamin 6. D-biotin 7.riboflavin
Column Dimensions: 150 x 4.6mm **Flow Rate:** 1.50ml/min **Mobile Phase:** A) 20mM KH_2PO_4 (pH 2.8)
B) 20mM KH_2PO_4 (pH 2.8) in 50:50 v/v MeOH/H_2O **Linear Gradient:** 20% to 70% B over 15 minutes
Temperature: 40°C **Detection:** UV, 205nm

These comparison chromatograms illustrate how the ACE C18-AR can achieve an adequate separation of all compounds in a sample when other C18 columns failed to do so.

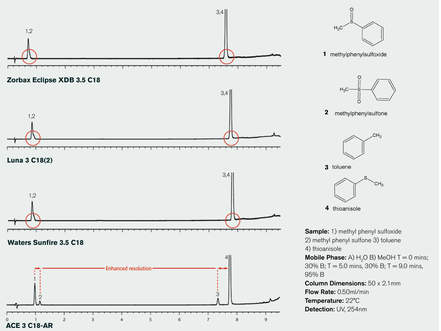
FIGURE 2: **Aromatic Nitrobenzenes**



Sample: 1) 1,3,5-trinitrobenzene 2) 1,3-dinitrobenzene 3) nitrobenzene 4) neutral molecule (reference)
Mobile Phase: 50:50 v/v MeOH/H_2O **Column Dimensions:** 150 x 4.6mm **Flow Rate:** 1.00ml/min **Temperature:** 40°C
Detection: UV, 210nm

The separation of aromatic nitrobenzenes offers an excellent example of how ACE C18-AR combines π-π selectivity of the phenyl functionality with the hydrophobic retention of the C18 chain to deliver quite a different separation from ordinary C18 phases. Notice how the elution order of the aromatic nitrobenzenes is reversed on ACE C18-AR compared to the other C18 phases, while the retention of the neutral marker is similar to other C18 phases.

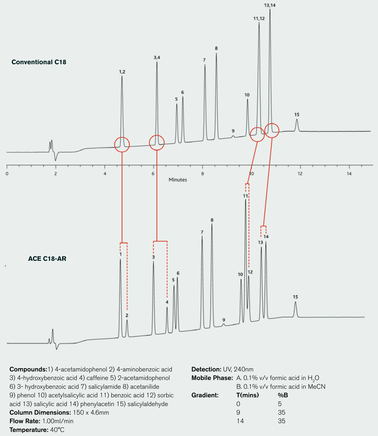
FIGURE 3: **Sulfur Compounds and Analogs**



Sample: 1) methyl phenyl sulfoxide 2) methyl phenyl sulfone 3) toluene 4) thioanisole
Mobile Phase: A) H_2O B) MeOH T = 0 mins; 30% B, T = 5.0 mins, 30% B; T = 9.0 mins, 95% B
Column Dimensions: 50 x 2.1mm
Flow Rate: 0.50ml/min
Temperature: 22°C
Detection: UV, 254nm

Conventional C18 columns are unable to separate all four compounds. ACE C18-AR, however, delivers an excellent separation of all compounds.

FIGURE 4: **Analgesics**



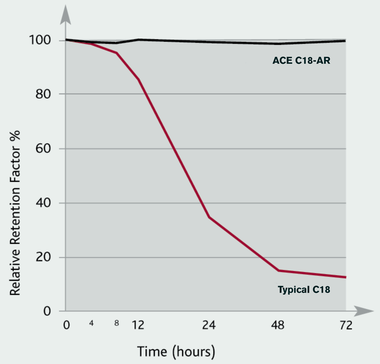
Compounds: 1) 4-acetamidophenol 2) 4-aminobenzoic acid 3) 4-hydroxybenzoic acid 4) caffeine 5) 2-acetamidophenol 6) 3- hydroxybenzoic acid 7) salicylamide 8) acetanilide 9) phenol 10) acetylsalicylic acid 11) benzoic acid 12) sorbic acid 13) salicylic acid 14) phenylacetin 15) salicylaldehyde
Column Dimensions: 150 x 4.6mm
Flow Rate: 1.00ml/min
Temperature: 40°C

Detection: UV, 240nm
Mobile Phase: A: 0.1% v/v formic acid in H_2O
B: 0.1% v/v formic acid in MeCN

Gradient:	T(mins)	%B
	0	5
	9	35
	14	35

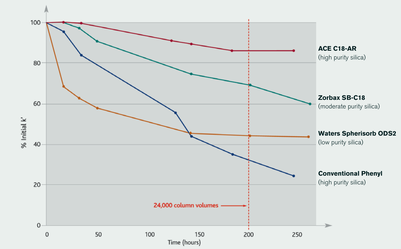
A conventional C18 is unable to resolve all compounds in this complex mixture of analgesics. Using the identical gradient conditions, ACE C18-AR provides adequate separation of all 15 analgesics due to its extra resolving power.

FIGURE 5: **Reproducible Chromatography with 100% Aqueous Mobile Phase**



As shown above, a typical C18 column will exhibit poor reproducibility over time when exposed to high (>95%) aqueous mobile phases. This is due to gradual exclusion of the polar mobile phase from the hydrophobic pores of the column. ACE C18-AR, with its integral phenyl functionality, however, is ideally suited for the analysis of polar analytes, even in 100% aqueous mobile phases.

FIGURE 6: **Accelerated Column Stability Study - 80°C at pH 1.9**



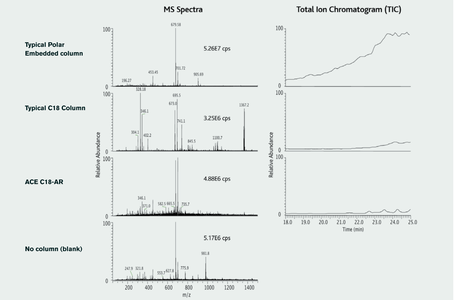
Acidic Exposure Conditions: **Mobile Phase:** 5:95 MeOH/0.1% TFA in H_2O (pH 1.9) **Flow Rate:** 0.20ml/min **Temperature:** 80°C
Column Dimensions: 50 x 2.1mm

Using conditions designed to accelerate column degradation, ACE C18-AR phase shows little retention loss, with lifetime equivalent to the highly robust ACE C18 phase. Both phases are manufactured from the same ultra pure silica, and outlast the Zorbax SB-C18, a phase previously recognized to provide excellent stability for high temperature and low pH applications.

As expected, a C18 bonded column based upon a low purity silica (Waters Spherisorb ODS2) shows a greatly reduced lifetime under these accelerated conditions.

Of particular note is the result comparing the lifetime of a conventional phenyl column to ACE C18-AR. Despite the use of a high purity silica, the lifetime of the phenyl column is diminished compared with ACE C18-AR, suggesting that ACE C18-AR may be suitable for applications in which phenyl columns are seen to exhibit reduced lifetime.

FIGURE 7: **ACE C18-AR Exhibits Low Column Bleed for LC/MS**

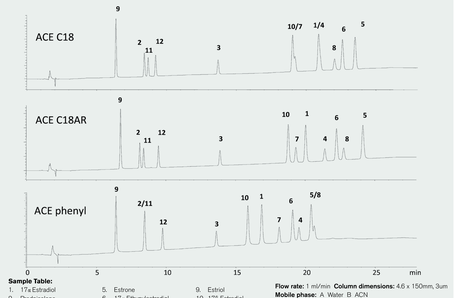


Column Dimensions: 150 x 3.0mm **Flow Rate:** 0.43ml/min **Temperature:** 60°C **Detection:** ESI mode (ThermoFisher Deca LCQ ion trap), full scan range 50 – 1500m/z **Mobile Phase:** A: 0.1% (v/v) formic acid in H_2O B: 0.1% (v/v) formic acid in MeCN

Gradient:	T(mins)	%B
	0	5
	20	90
	26	90
	26	5

The TIC trace and MS spectra for the polar embedded column (previously seen to show significant bleed by UV detection) again shows a high level of column bleed when analyzing by LC/MS. The MS spectra from a blank run (performed with no column attached) enables the background system bleed to be quantified. Both the ACE C18-AR column and a typical C18 column exhibit bleed levels similar to the blank run, denoting that negligible column bleed is occurring.

FIGURE 8: **Steroids on Different Bonded Phases**



Sample Table:
1. 17α Estradiol
2. Prednisolone
3. Corticosterone
4. 19-Norethindrone
5. Estrone
6. 17α Ethynylestradiol
7. Cortisone 21-acetate
8. 21-Hydroxyprogesterone
9. Estriol
10. 17β Estradiol
11. Hydrocortisone
12. Cortisone

Flow rate: 1 ml/min **Column dimensions:** 4.6 x 150mm, 3um
Mobile phase: A Water B ACN
Gradient: Time (min)%B

0	25
24	46
26	46
27	25

Wavelength: 214 nm **Temperature:** 20°C

The above chromatogram illustrates the separation of a complex mixture of steroids on three unique bonded phases. Since all columns are bonded to the same silica, only the bonded phase accounts for the differences in resolution. Only ACE C18-AR is capable of resolving all steroids through a combination of hydrophobic and pi - pi interactions with the bonded phase.

Conclusion

ACE® C18-AR is an excellent choice to begin any reversed phase method development project. It provides all of the benefits of C18, with additional selectivity for polar compounds.

® ACE is a registered trademark of Advanced Chromatography Technologies (ACT).