Exceptional Selectivity of Agilent ZORBAX Eclipse Plus Phenyl-Hexyl Columns to Separate Estrogens

Application Note

Pharmaceutical, Environmental

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Abstract

A new ZORBAX stationary phase, Agilent ZORBAX Eclipse Plus Phenyl-Hexyl, separated five estrogen type steroids better than a C18 and better than three other phenyl columns under identical conditions, due to its unique selectivity for analytes containing phenyl groups. The comparisons support selectivity as the most important factor to optimize resolution, to separate peaks completely. Selectivity can be quickly altered by simply substituting columns with different phases; therefore, having a variety of columns (stationary phases) available for method development improves the chances of discovering the best HPLC method. Eclipse Plus Phenyl-Hexyl characteristics such as improved silica, bonding technology, and molecular interactions of the phenyl-hexyl moiety with aromatic groups of the analytes make the Eclipse Plus Phenyl-Hexyl column valuable for a method developer's column collection.

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Introduction

Most reversed phase LC method development protocols include changing the organic portion of the mobile phase, usually between methanol and acetonitrile, changing the pH of the aqueous portion of the mobile phase if analytes are ionic, and of course running mobile phase gradients. Other variables such as temperature and mobile-phase ionic strength can also be effective for changing resolution. Substituting columns is also the norm, as more stationary phases are commercially available, and automated instrumentation, including column switching valves and LC method development software, make column investigations easy [1].

To achieve the best resolution and develop the best method it is important to try a variety of stationary phases, because selectivity is the dominant factor in the resolution equation [2]. Changes in the selectivity can improve resolution more than adding efficiency (N) or retention (k').

There are several reasons why Eclipse Plus Phenyl-Hexyl columns are a great complement to the method developer's column portfolio for maximizing selectivity differences via column substitution. Eclipse Plus columns achieve superior performance by using ultrapure fully hydroxylated silica and proven bonding technology. The bonding process tightly controls the ligand and endcapping density of the sol-gel totally porous particle. The phenyl group of the stationary phase has enhanced molecular interactions with aromatic analytes, providing an additional retention mechanism and enhanced selectivity. Furthermore, the hexyl linkage of the stationary phase also plays a significant role in the overall retention characteristics of the stationary phase.

Here we demonstrate the useful selectivity of ZORBAX Eclipse Plus Phenyl-Hexyl compared to ZORBAX Eclipse Plus C18 for estrogens that have phenyl groups. While it is no great leap to conclude that these two distinct stationary phases could indeed have different selectivity, we also compared the Eclipse Plus Phenyl-Hexyl to other phenyl columns and found similar changes in resolution. While the phenyl-hexyl phase was an excellent choice here, a variety of stationary phases, including alkyl (C18, C8, and C3), phenyl, and other phase types (for example, cyano, amide linked) are advantageous to try in method development protocols, because stationary phase selectivity is hard to predict due to unique requirements of each method, and therefore, best determined by experimentation.

Experimental

Column comparisons were performed on the Agilent 1200 Rapid Resolution LC (RRLC) system:

- G1312B binary pump with mobile phase A: water, B: methanol; flow rate: 1 mL/min (40:60 A:B)
- G1376C automatic liquid sampler (ALS), injection volume 3 µL
- G1316B thermally controlled column compartment, 25 °C
- G1315C diode array detector (DAD) at 220 nm, with a G1315-60025 flow cell (6-mm path, 5-µL volume), response time setting of 1 s

Columns:

- ZORBAX Eclipse Plus Phenyl-Hexyl 4.6 mm × 100 mm, 5 µm, PN 959996-912
- ZORBAX Rapid Resolution Eclipse Plus Phenyl-Hexyl 4.6 mm × 100 mm, 3.5 µm, PN 959961-912
- ZORBAX Rapid Resolution HT Eclipse Plus Phenyl-Hexyl 4.6 mm × 100 mm, 1.8 µm, PN 959964-912
- ZORBAX Rapid Resolution Eclipse Plus C18 4.6 mm × 100 mm, 3.5 µm, PN 959961-902
- Unnamed commercial phenyl columns were all 4.6 mm x 100 mm, 5 µm, spherical silica

  Columns from other suppliers were not available in 3.5- or 1.8-µm sizes, therefore the 5-µm column was used for direct comparisons of selectivity.

Finger-tight fittings (Agilent PN 5065-4426 [10/pk]) were used to easily install and uninstall the columns to the temperature-controlled column compartment and directly to the DAD flow cell.

Vials: Amber screw cap (Agilent PN 5182-0716)

Vial caps: Blue screw cap (Agilent PN 5282-0723)

The five estrogen-related analytes were obtained from Sigma-Aldrich (St. Louis, MO) and dissolved in MeOH to a concentration of about 0.5 mg/mL. A composite sample was then made by combining 50 µL aliquots of each individual estrogen solution and then diluting the 250 µL mixture with 750 µL water. The analyte names and structures are shown in Figure 1.

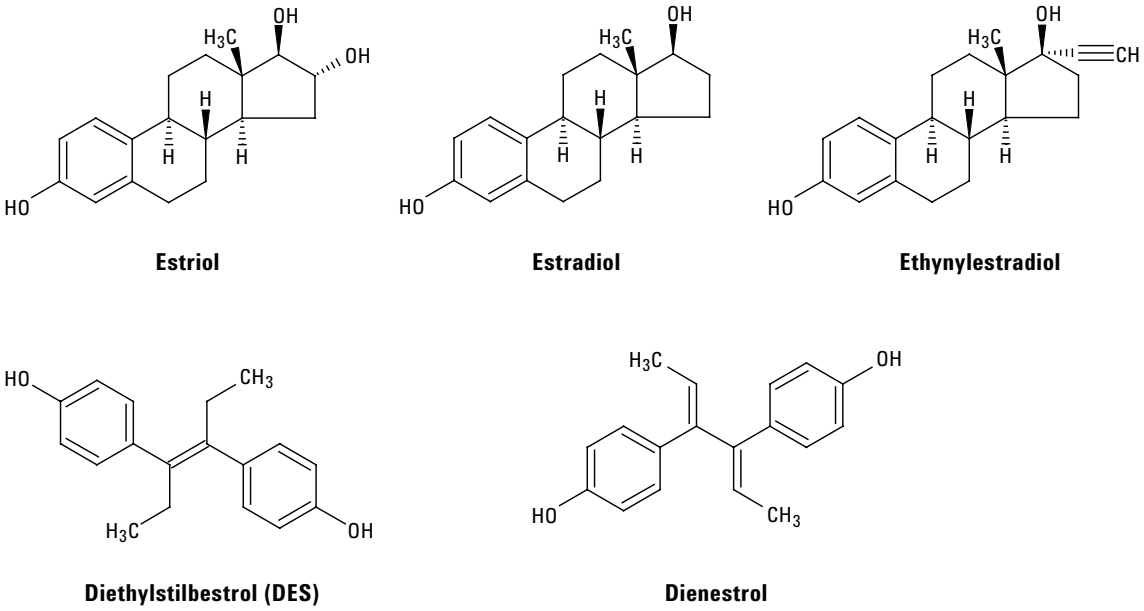

Figure 1. Estrogens analyzed in this study are heavily conjugated.

Results and Discussion

To compare stationary phases, all chromatographic parameters were held constant, except column choice. Methanol was used instead of acetonitrile as the organic modifier, due to enhanced molecular interactions of phenyl groups in methanol [3]. Isocratic mobile phase composition was 60% methanol and 40% water.

Although not always true, an alkyl phase, such as C18, can be expected to exhibit different selectivity and ultimately different resolution compared to a phenyl phase. Figure 2 demonstrates this. More interesting, however, is that the C18 column has less resolution despite being made from smaller particles (5 vs. 3.5 µm). The smaller particles produce more efficiency in the same length column. Despite the C18 column's significant advantage in efficiency, it is not enough to overcome the selectivity advantage that the phenylhexyl column has for the estrogens.

The ideal efficiency (N) of the column can be calculated by:

$N = L/H$

The length of the column (L) divided by the height equivalent of a theoretical plate, H (roughly equal to the length of two particle diameters). The 5-µm column therefore has a theoretical efficiency of about N = 100,000 µm/(2) (5 µm) = 10,000. The Rapid Resolution (3.5 µm) column has N = 14,300, or 30% more efficiency.

Based on Figure 2, the Eclipse Phenyl-Hexyl is the better option. A 3.5- or 1.8-µm Eclipse Plus Phenyl-Hexyl column could then be used to further optimize the method in two ways (data not shown): One, use smaller particles in the same length column to provide the benefits from higher efficiency (narrower bands, higher sensitivity, and resolution), or two, use the smaller particles in a shorter column to shorten the analysis time while maintaining chromatographic performance.

One might predict that selectivity differences between various phenyl columns is less obvious compared to a C18 column, but for this estrogenic compounds analysis, we found selectivity differences just as pronounced. The estrogen analysis on four different phenyl columns is shown in Figure 3. Recall that Figure 2 showed that the Eclipse Plus C18 partially resolved all five estrogens; however, in Figure 3, two phenyl columns under the same conditions, separate only four of the five. Just as all C18 columns are not alike, the same is true with phenyl columns. Key differences include silanol endcapping, phenyl linkage (for example, hexyl, ethyl, and propyl), % carbon load, and silica characteristics. The combination of these properties found in Eclipse Plus Phenyl-Hexyl produced the best resolution of the four phenyl columns for the estrogens.

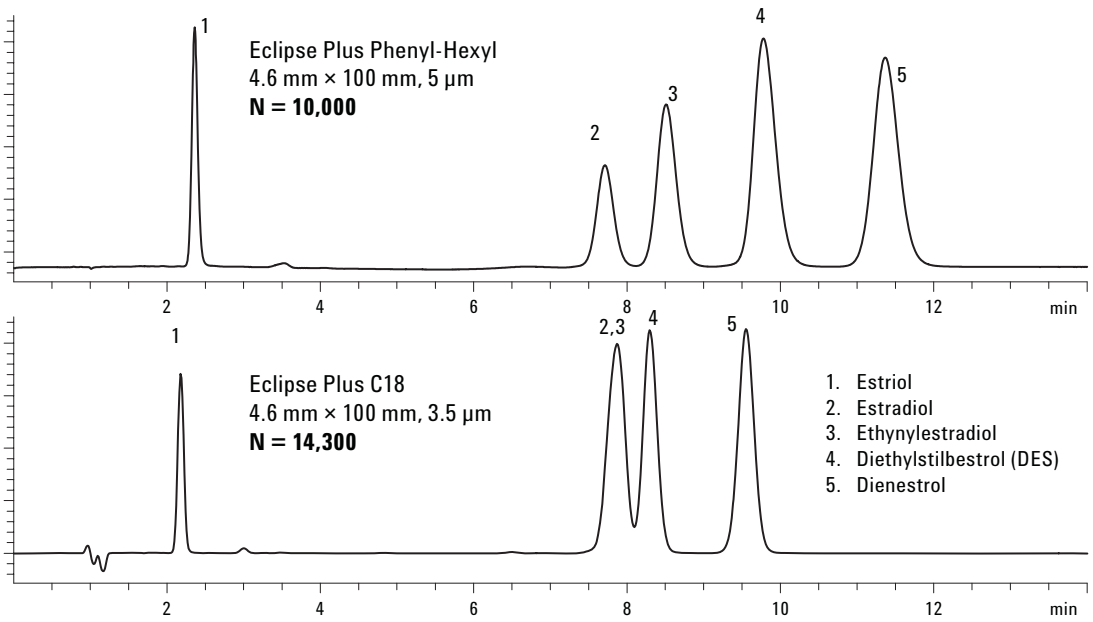

Figure 2. The 3.5-µm column has 30% more efficiency, but column selectivity has a greater effect on resolution.

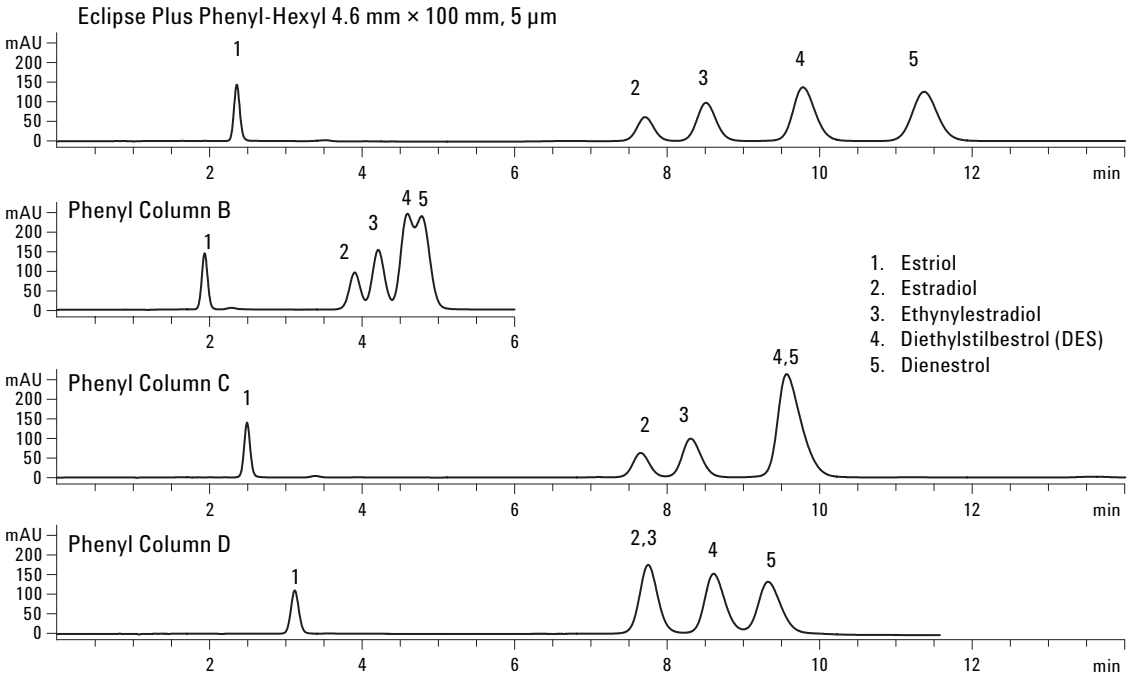

Figure 3. Different selectivity among phenyl columns.

Conclusions

The Eclipse Plus series of HPLC columns, including the Eclipse Plus Phenyl-Hexyl column, have improved silica manufacturing and bonding technology and are great columns to alter selectivity and quickly improve a chromatographic separation. Predicting selectivity differences among stationary phases is often not straightforward so the optimum stationary phase should be determined experimentally. The Eclipse Plus Phenyl-Hexyl column was shown to have a considerable difference in selectivity compared to an Eclipse Plus C18 column, but just as much a difference when compared to several other commercially available phenyl-type columns for an analysis of estrogens. In addition to the improved silica manufacturing and bonding technology, Eclipse Plus Phenyl-Hexyl's unique molecular interactions of the phenyl-hexyl ligand with the aromatic estrogens made Eclipse Plus Phenyl-Hexyl the best column choice for this estrogen analysis.

References

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