APPLICATIONS NOTE

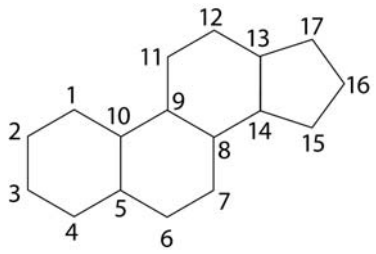
Improved HPLC Analysis of Steroids

Using Restek's Unique Allure™ Biphenyl Column

Background

Steroids are a unique class of compounds in that all structural variation is centered on a specific conjugated ring structure. The basic, saturated, steroid structure is referred to as perhydrocyclopentanophenanthrene—a cyclopentane ring linked to phenanthrene (Figure 1). From this structure, various levels of unsaturation (double bonds) and diverse ring substituents (functional groups) create numerous variations which, in turn, produce much diversity in the biological effects of the steroids.

Figure 1 Perhydrocyclopentanophenanthrene base structure of steroid molecules.



Groups of endogenous steroids produced by the human body are formed biochemically from a common metabolic precursor and, therefore, have similar chemical structures. Cholesterol is the common metabolic precursor of the sex hormones, for example. The chemical structures of synthetically produced steroids intentionally mimic the chemical structures of the endogenous steroids. The end result is that steroids, whether endogenous or synthetic, can have very different functions, but they share a similar chemical structure. Because the diversity in steroid composition consists of functional group and ring variations around a consistent structure, we chose steroids to test HPLC stationary phase selectivity: their systematic chemical variation provides a traceable model for determining the effects of ring unsaturation on chromatographic behavior.

In the basic steroid structure, certain carbon positions contribute more often than others to structural variation. Carbons in position 1 through 5 often participate in double bonding, for instance, and carbon 17 often has one of a variety of functional groups. By concentrating on these dynamic positions in the steroid structure, the effects of altering ring unsaturation and functional group identity and position can be used to determine and predict chromatographic behavior of HPLC stationary phases.

Column Chemistry

Reversed phase HPLC commonly is used to identify and quantify steroids. Because steroids are hydrophobic molecules, they typically can be analyzed adequately using a traditional hydrophobic reversed phase packing, such as a C18 packing. The aliphatic hydrocarbon chain of the C18 stationary phase resolves target compounds primarily through hydrophobic interactions. Through this mechanism, hydrophobic compounds, such as steroids, are more attracted to the hydrophobic stationary phase than to the mobile phase. Resolution is achieved as the most hydrophobic molecules interact most with the hydrophobic phase. Another mechanism for analyzing steroids is polar interaction. Polar interaction relies on dipole-dipole or dipole-induced dipole interactions: a permanent dipole in the stationary phase interacts with polar molecules or repels negatively charged electron clouds in nonpolar molecules. A cyano stationary phase can use this separation mechanism, producing a selectivity different from that of C18 (or other) alkyl chain stationary phases. A third, and more selective, mechanism for separating steroidal structures is via pi-pi (π-π) interactions. π-π interactions occur when unsaturation—i.e., double or triple bonds—is present. With steroids, for example, π-π interactions can occur when double bonds in the steroid molecule and the stationary phase overlap. Phenyl stationary phases mainly use this type of interaction. Relative to traditional phenyl stationary phases, the Allure™ Biphenyl stationary phase comprises a higher concentration of phenyl groups, in a sterically favorable configuration, and, therefore, is capable of establishing more numerous π-π interactions.

Results

We first used an analysis of seven corticosteroids (Figure 2) to determine the comparative effectiveness of various stationary phases for separating related steroidal structures. To limit operational bias, we performed all analyses with the same HPLC instrumentation and under the same isocratic conditions. The Allure™ Biphenyl phase and the C18 phase were the most selective stationary phases for a seven-steroid mixture (Figure 3). The Allure™ Biphenyl phase demonstrated the greatest retention and selectivity and was the only phase capable of resolving all test compounds in a simple isocratic analysis.

When the structures of hydrocortisone, cortisone, and prednisone are compared to the chromatographic results, the reason for the Allure™ Biphenyl phase's superior performance is revealed. Hydrocortisone and prednisone have identical configuration at position 17, but differ in ring unsaturation (Figure 2), whereas hydrocortisone and cortisone have nearly identical ring unsaturation, but differ in position 17 functional group orientation. Cortisone is resolved to baseline by the C18 stationary phase, but hydrocortisone and prednisone coelute (Figure 3). Thus, the C18 phase is selective for differences in functional group orientation, but not for subtle differences in hydrocarbon ring unsaturation. The Allure™ Biphenyl stationary phase resolves hydrocortisone and prednisone almost to baseline, indicating that this phase can better resolve compounds with differences in ring unsaturation.

Figure 2 Differences in saturation within the carbon rings and the identity and position of functional groups create numerous structural variations among steroids. Hydrocortisone, cortisone, and prednisone exhibit only slight structural differences.

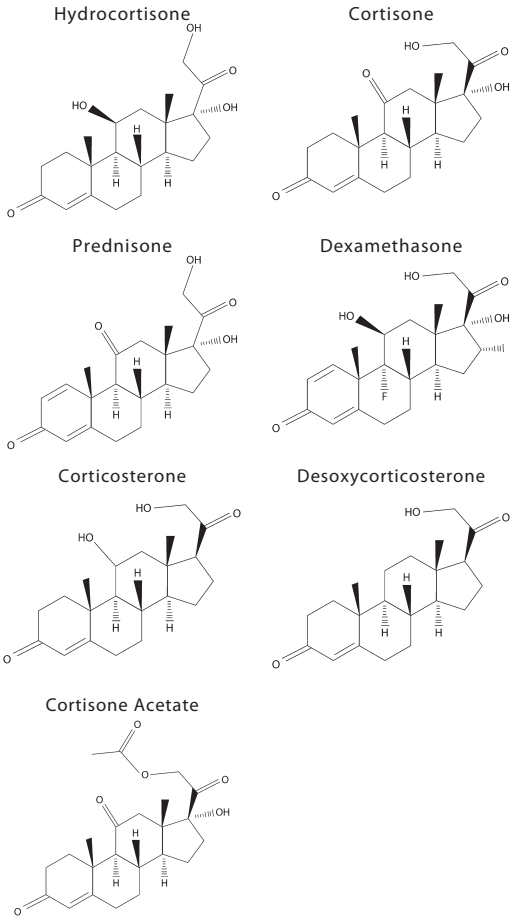
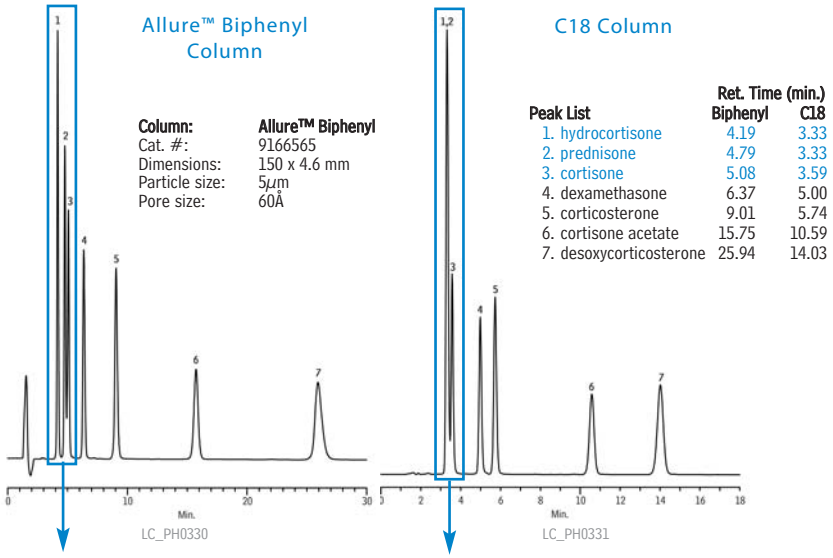
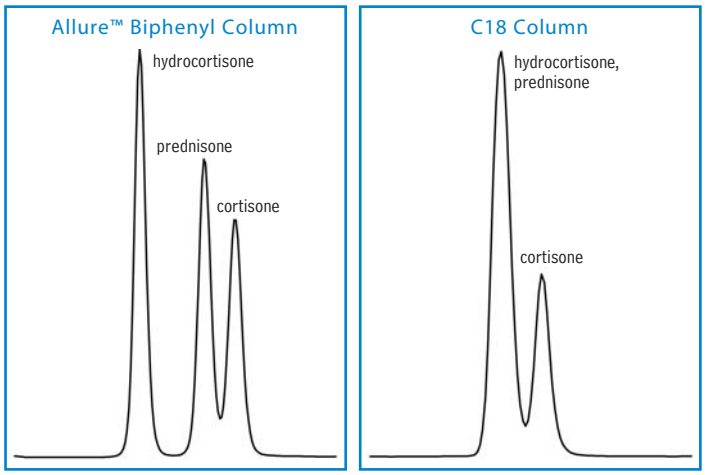
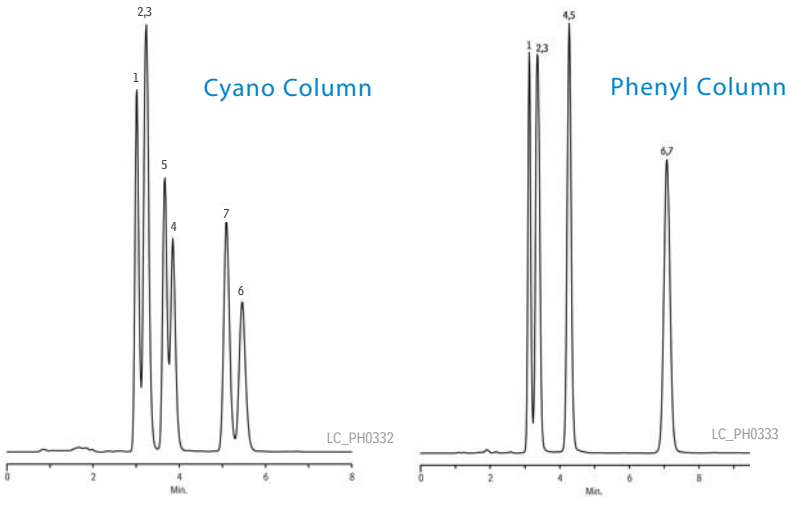


Figure 3 Separation of corticosteroids is superior on the Allure™ Biphenyl phase.





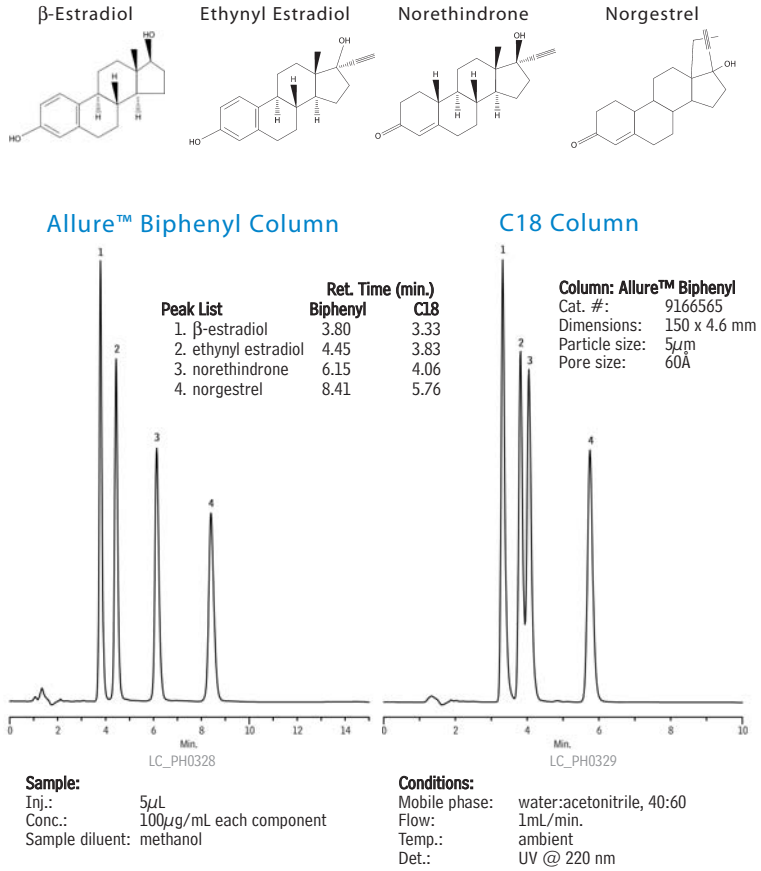


Sample:
Inj.: 5µL
Conc.: 100µg/mL each component
Sample diluent: methanol

Conditions:
Mobile phase: water:acetonitrile, 60:40
Flow: 1mL/min.
Temp.: ambient
Det.: UV @ 254 nm

To further explore this distinction between the Allure™ Biphenyl stationary phase and C18 stationary phases, we made a direct comparison of their resolving power, using an analysis of contraceptives. β-estradiol, ethynyl estradiol, and norethindrone (Figure 4) show differences and similarities in functional group and ring structures comparable to those of the corticosteroids. As expected, the C18 phase completely resolved β-estradiol and ethynyl estradiol (Figure 4), which differ in the orientation and structure of the position 17 ethynyl functional group, but the phase could not resolve ethynyl estradiol and norethindrone, which have identical position 17 functional groups, but small differences in hydrocarbon ring unsaturation. Overall, again, retention and selectivity were markedly better for the Allure™ Biphenyl phase, which resolved all three compounds to baseline. Most important, the Allure™ Biphenyl phase showed greater resolving capability for unsaturation differences in the hydrocarbon ring structure, as noted by the superior resolution of ethynyl estradiol and norethindrone.

Figure 4 Baseline resolution of contraceptive steroids on an Allure™ Biphenyl column.



HPLC Tech Tips Wall Chart

(lit. cat.# 59894A)

Almost everything you need to remember about HPLC, condensed into 3 feet by 2 feet: mobile phase basics, buffers (types, pKas, pH ranges, formula masses, more), miscibility and solubility chart (invaluable!), system setup and optimization, detector tips, pressure conversion factors, most-used chromatographic equations, column storage essentials. Post near your instrument to save time; perhaps save a column.

Call us or visit our website for your free wall chart.

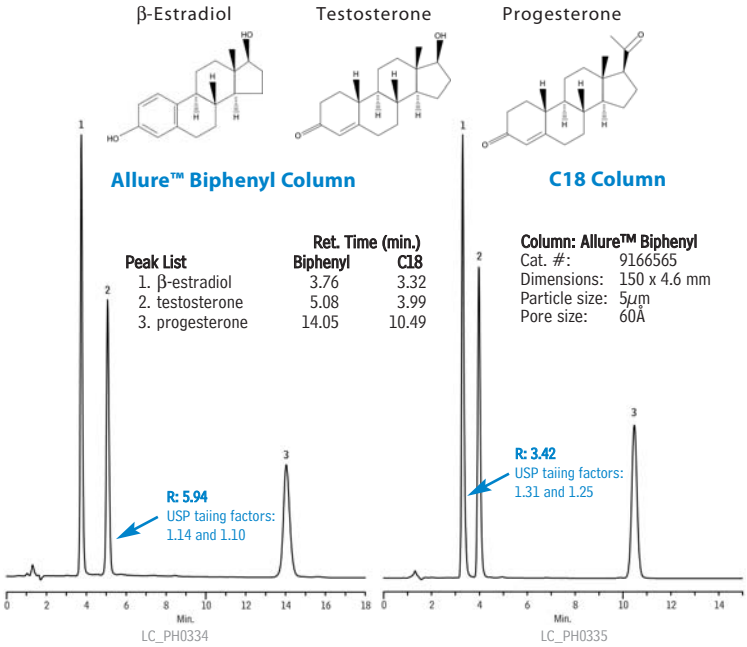
Finally, to determine the reproducibility of the Allure™ Biphenyl phase's enhanced selectivity for differences in hydrocarbon ring unsaturation, we analyzed three endogenous steroid hormones, β-estradiol, testosterone, and progesterone, under the same isocratic conditions as the contraceptives. The chemical structures of β-estradiol and testosterone contain significant differences in ring double bonding (Figure 5). Therefore, by comparing resolution of these two compounds on the Allure™ Biphenyl and C18 phases (Figure 5), we can confirm the correlation between hydrocarbon ring variations and resolution. The C18 column produced a resolution of 3.42 between β-estradiol and testosterone, with USP tailing factors of 1.31 and 1.25, respectively; the Allure™ Biphenyl column provided a resolution of 5.94—a 43% increase—and superior tailing factors of 1.14 and 1.10. These data indicate the Allure™ Biphenyl phase exhibits better efficiency, as well as better selectivity.

Conclusions

The steroid analyses discussed here demonstrate that, through π- π interactions, the Allure™ Biphenyl stationary phase offers a unique and effective alternative to hydrophobic interaction or polar interaction for resolving compounds with unsaturation differences in their hydrocarbon ring structure. π- π interactions offer better retention, selectivity, and efficiency for resolving such compounds. Moreover, when position and unsaturation differences are present solely in the functional groups, and not in the hydrocarbon ring structure, the Allure™ Biphenyl stationary phase appears to offer many of the same characteristics available in a traditional C18 stationary phase.

The superior selectivity and efficiency exhibited by the Allure™ Biphenyl stationary phase should make it a well suited choice for developing and validating pharmaceutical methods, especially stability-indicating methods. The superior selectivity could provide better specificity and resolution between an analyte, its degradation products, synthesis intermediates, and process impurities. The superior efficiency can afford tighter, more reproducible system suitability parameters.

Figure 5 Endogenous steroids confirm the superior performance of Allure™ Biphenyl columns.



Sample:
Inj.: 5µL
Conc.: 100µg/mL each component
Sample diluent: methanol

Conditions:
Mobile phase: water:acetonitrile, 40:60
Flow: 1mL/min.
Temp.: ambient
Det.: UV @ 220 nm

free literature

Genuine Restek Replacement Parts for HPLC Systems
(lit. cat.# 59012A*)

This publication lists a wide selection of Restek parts for Agilent, Beckman, Hitachi, PerkinElmer, Shimadzu, Thermo Separations Products, and Waters instruments, to keep these systems running smoothly and chromatography sharp. Genuine Restek Replacement Parts equal or exceed the performance of original equipment components.

Innovative Chromatography Columns and Supplies
(lit. cat.# 580021*)

The 2006 Restek chromatography products catalog features new GC and HPLC columns, new tools and accessories, new sample collection and sample preparation products, new reference chemical mixes, and new applications chromatograms, plus an extensive inventory of replacement parts for chromatographs, proprietary surface treatments for deactivating and protecting sample pathways, and more.

Allure™ Biphenyl

Physical Characteristics:

particle size: 3µm or 5µm, spherical
pore size: 60Å
carbon load: 23%
endcap: yes
pH range: 2.5 to 7.5
temperature limit: 80°C

Chromatographic Properties:

Highly retentive and selective phase for unsaturated compounds. Greater retention than phenyl phases; uses high-purity, Type B silica.

Length	1.0mm ID cat.#	2.1mm ID cat.#	3.2mm ID cat.#	4.6mm ID cat.#
5µm Columns				
30mm	9166531	9166532	9166533	9166535
50mm	9166551	9166552	9166553	9166555
100mm	9166511	9166512	9166513	9166515
150mm	9166561	9166562	9166563	9166565
200mm	9166521	9166522	9166523	9166525
250mm	9166571	9166572	9166573	9166575

To order a 2.1mm, 3.2mm, or 4.6mm ID column with a Trident™ Integral Inlet Fitting, add "-700" to the catalog number for the column.
Example: 100mm x 4.6mm ID Allure™ Biphenyl column with Trident™ Integral Inlet Fitting: 9166515-700 (Nominal additional charge.)
Also order an XG-XF fitting (cat.# 25026 or25062) and guard cartridges from our catalog.

*When requesting literature from outside the US, please add "-INT" to the lit. cat.#.