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Evaluation of Newer HPLC and UHPLC Column Technologies for the Analysis of Steroids

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This study evaluates the performance of five different HPLC/UHPLC columns used to quantitatively screen steroids using HPLC/UV. The goal of the study was to dramatically reduce conventional analysis times while maintaining baseline resolution between 9 steroids. This work resulted in a method that provided run times of under 6 minutes and excellent separation of 9 steroids, all of which can be attributed to the technology behind the Kinetex 2.6 µm XB-C18 core-shell HPLC/UHPLC column.

Introduction

Increased testing, coupled with the migration of ELISA based analysis to HPLC based assays, is forcing chromatographers to make improvements to existing methods. Run times of 20 to 30 minutes will no longer suffice for the throughput demands of broad range quantitative steroid analyses. Therefore, the goal of this study is to screen the latest in column technology in search of a faster and more selective steroid HPLC/UV application as compared to conventional approaches.

Materials and Methods

HPLC analyses were performed using an HP 1200 LC system (Agilent Technologies, Palo Alto, CA, USA) with an upper pressure limit of 600 bar, equipped with a UV DAD detector.

The HPLC columns that were screened included:

1. Kinetex XB-C18 2.6 µm, (Phenomenex, Torrance, CA, USA)
2. ACQUITY® BEH C18 1.7 µm, (Waters Corp., Milford, MA, USA)
3. ACQUITY CSH™ C18 1.7 µm, (Waters Corp., Milford, MA, USA)
4. XSelect™ CSH C18 3.5 µm, (Waters Corp., Milford, MA, USA)
5. HALO® C18 2.7 µm, (Advanced Materials Technology, MA, USA)

Results and Discussion

Steroid analysis performed by LC/UV can be challenging because UV detection requires adequate separation in order to be used for quantitative analysis. Furthermore, steroids are related in terms of chemical structure and are relatively non-polar, making it difficult to achieve chromatographic resolution. To be considered baseline resolved, an R_s of ≥ 1.5 between neighboring peaks must be reached. This value can be calculated by using the resolution equation:

$$R = 2(t_{R1} - t_{R2})/W_{b1} - W_{b2}$$

Historically, to maintain peak resolution of $R_s \geq 1.5$ using traditional 5 µm fully porous HPLC medias, long columns and subsequently long methods were required for steroid analysis via LC/UV. It has been determined that these long analysis times are due to the low efficiency, low resolving power of larger particle, fully porous media. In an effort to achieve faster run times and more resolved chromatograms, this study was performed to evaluate the performance of several core-shell and sub-2 µm HPLC/UHPLC media (**Figures 1** through **5** and **Table 1**).

Figure 1.
Steroids by LC/UV using Kinetex XB-C18 2.6 µm, 50 x 2.1 mm, 235 bar

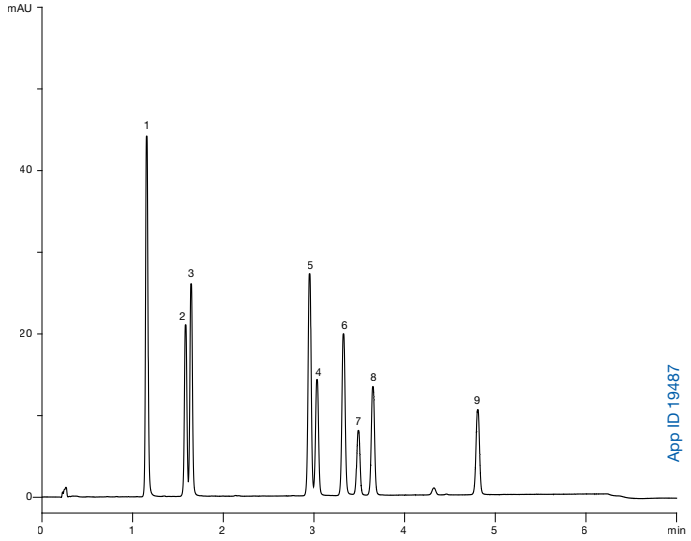

Figure 2.
Steroids by LC/UV using ACQUITY BEH C18 1.7 µm, 50 x 2.1 mm, 386 bar

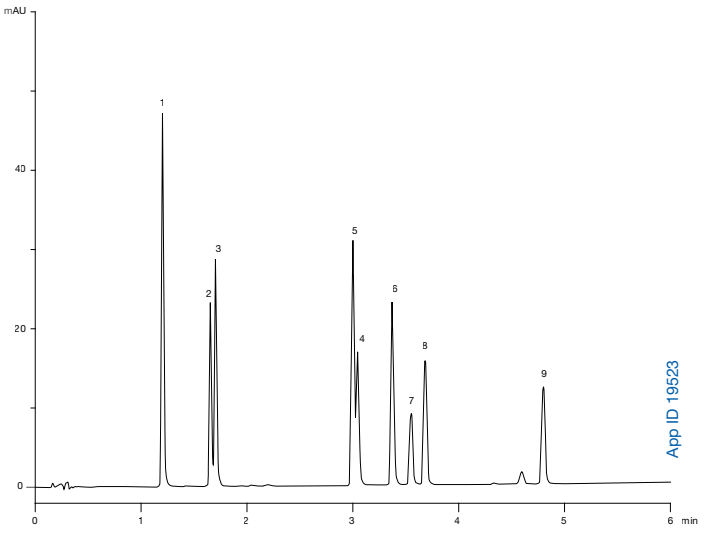

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Figure 3.
Steroids by LC/UV using ACQUITY® CSH™ C18 1.7 µm, 50 x 2.1 mm, 414 bar

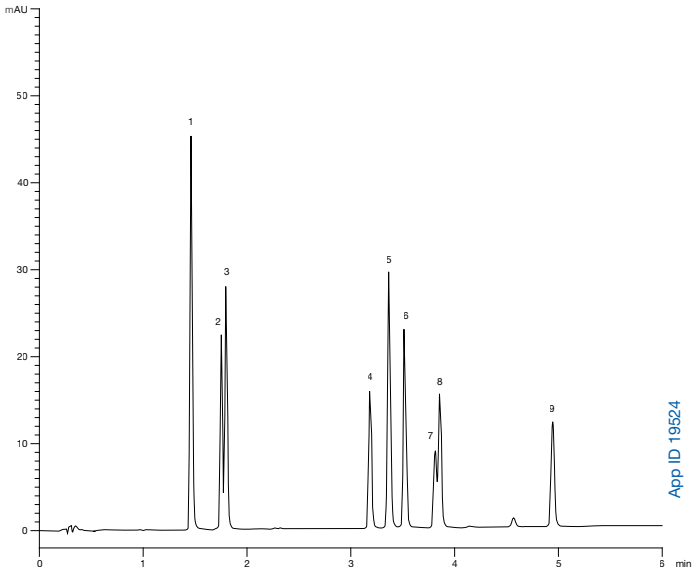


Figure 4.
Steroids by LC/UV using XSelect™ CSH C18 3.5 µm, 50 x 2.1 mm, 131 bar

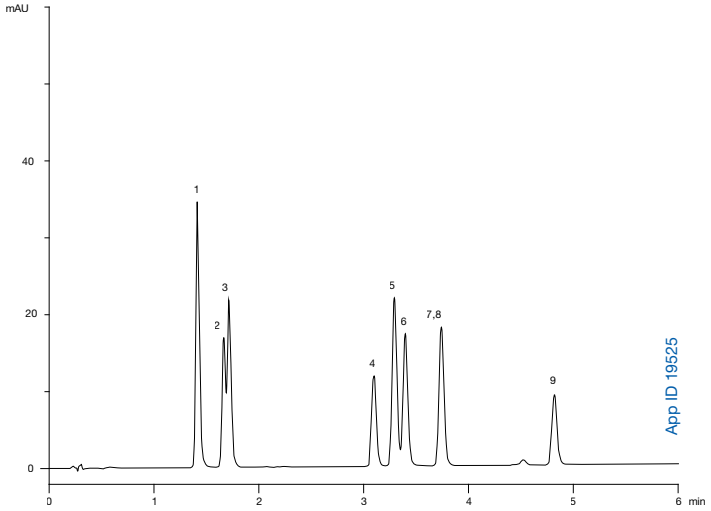
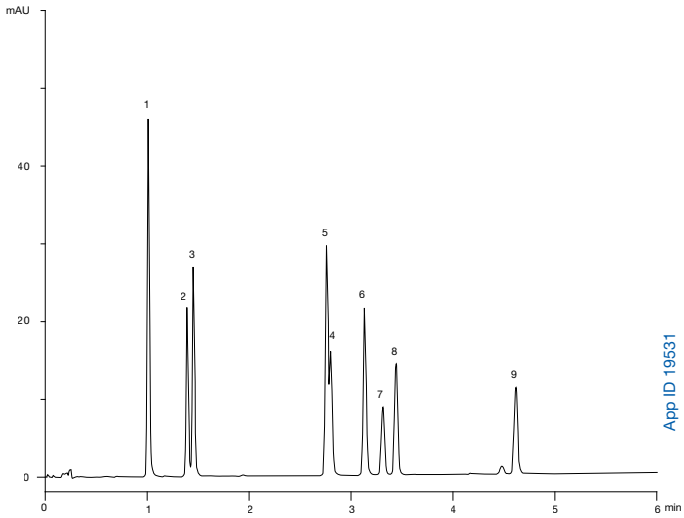


Figure 5.
Steroids by LC/UV using HALO® C18 2.7 µm, 50 x 2.1 mm, 189 bar



LC/UV

All column dimensions were 50 x 2.1 mm.
HPLC conditions were identical for all columns.
All analytes were supplied in methanol and were each present at a concentration of 200 µg/mL.

Mobile Phase: A: Water
B: Acetonitrile

Gradient:

Time (min)	% B
0.00	20
6.00	60
6.01	5
8.00	5

Flow Rate: 0.5 mL/min
Column Temperature: 30 °C
Detection: UV @ 220 nm
Sample:
1. Estriol
2. Hydrocortisone
3. Cortisone
4. Cortisone-21-acetate
5. Estradiol
6. 21-Hydroxyprogesterone
7. Estrone
8. 17-Hydroxyprogesterone
9. Deoxycorticosterone

Table 1.
R_s values of critical pairs

Column	R_s (Analytes 2,3)	R_s (Analytes 4,5)	R_s (Analytes 7,8)
Kinetex® XB-C18 2.6 µm	1.52	1.60	2.89
ACQUITY BEH C18 1.7 µm	1.29	0.84	2.67
ACQUITY CSH C18 1.7 µm	1.15	3.79	0.79
XSelect CSH C18 3.5 µm	0.73	2.53	0.00
HALO C18 2.7 µm	1.54	0.74	2.39

* Red coloration denotes unresolved critical pairs

In order to achieve efficient separation of the 9 steroids analyzed in under 6 minutes, excellent efficiency and unique selectivity must be combined for maximum resolving power.

As shown in **Figures 1** through **5** and **Table 1**, the Kinetex XB-C18 core-shell HPLC/UHPLC column gave the best performance for this separation. In fact, the Kinetex XB-C18 was the only column of those tested that was able to achieve complete baseline resolution of all critical pairs involved.

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The two fully porous sub-2 µm columns evaluated, ACQUITY® BEH C18 and ACQUITY CSH™ C18, were each only able to resolve one of the critical pairs. Sub-2 µm columns are implemented for high efficiency but even so, it was not enough to separate the compounds analyzed in this study. It was also seen that the larger particle size 3.5 µm XSelect™ CSH C18 HPLC column yielded similar results as the 1.7 µm counterpart.

The other core-shell HPLC column in this screen, HALO® C18, fared better than the three fully porous particle columns but was still unable to separate peaks 4 and 5.

The improved performance of the Kinetex® XB-C18 column may be due to the technology incorporated into the core-shell particle design. The Kinetex technology is comprised of a nearly monodispersed 1.9 µm solid silica core and a 0.35 µm porous silica shell. This particle design results in a very tight particle size distribution, as seen in **Figure 7**, and a more homogeneous packed column bed, which significantly reduces peak dispersion due to eddy diffusion (the "A" term of the van Deemter equation) (**Figure 6**). Additionally, the short diffusion path of the 0.35 µm porous silica shell allows for faster kinetics of diffusion, thereby minimizing peak dispersion due to resistance to mass transfer (the "C" term in the van Deemter equation) (**Figure 8**).

Figure 6.
Effect of Eddy Diffusion

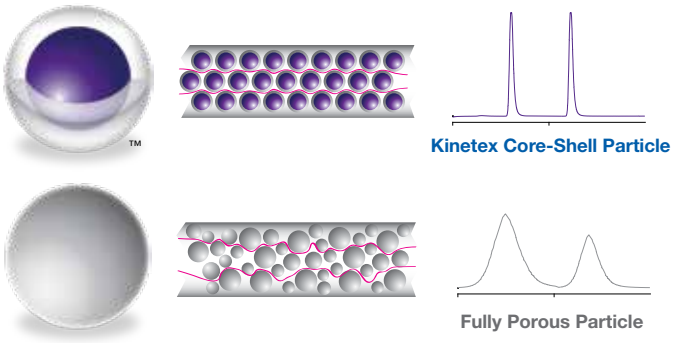


Figure 7.
Uniform Particle Size Distribution

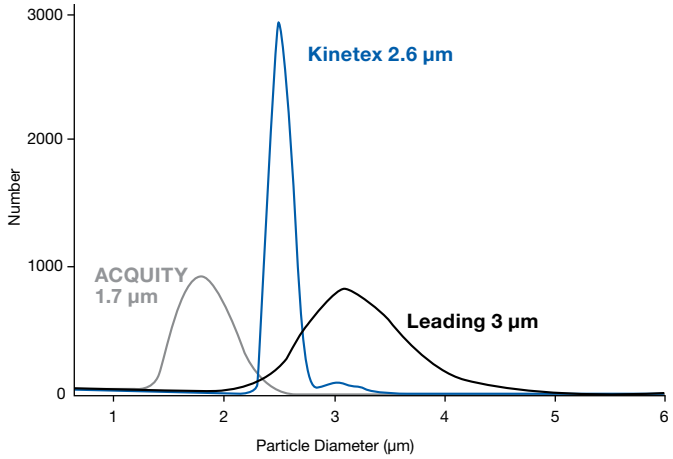


Figure 8.
Increased efficiency through faster mass transfer

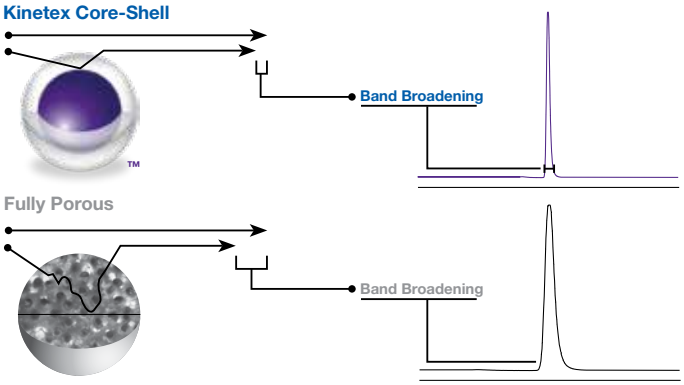
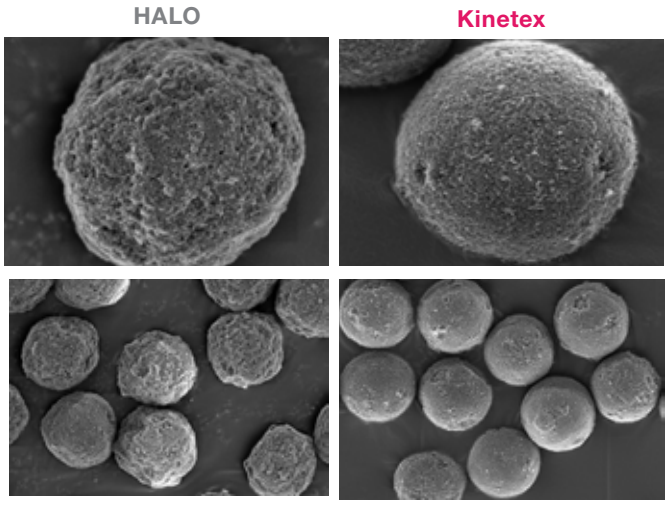


Figure 9 shows an SEM image of the Kinetex core-shell particle compared to the HALO core-shell particle. Kinetex provides a smoother, more uniform particle while the HALO particle is less consistent in terms of uniformity and smoothness. This difference between the two particles may account for the improved performance seen with the Kinetex column due to improved mass transfer and decreased band broadening due to eddy diffusion.It should

Figure 9.
Electron micrographs comparing Kinetex core-shell media to HALO core shell media. Kinetex media is shown on the right. Note the spherical shape and improved surface smoothness of the Kinetex media.



also be noted that this separation on the Kinetex XB-C18 column was performed at 235 bar, which makes it easily amenable to virtually every HPLC system while the sub-2 µm columns result in backpressures close to or exceeding 400 bar.

Conclusions

Meeting the increased demands of the ever-evolving clinical diagnostic industry is an on-going challenge. In response, this study provides a solution that can successfully separate 9 steroids in under 6 minutes with back pressure compatible with a conventional LC/UV system using a Kinetex XB-C18 core-shell HPLC column. This solution allows all labs, including those that are not equipped with an LC/MS system, to generate excellent chromatographic results in less time. This allows labs to provide quicker turnaround and reporting times than what was previously possible with fully porous HPLC media.

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Ordering Information

Kinetex® 2.6 µm Minibore Columns (mm)						SecurityGuard ULTRA Cartridges‡
Phases	**30 x 2.1**	**50 x 2.1**	**75 x 2.1**	**100 x 2.1**	**150 x 2.1**	**3/pk**
EVO C18	00A-4725-AN	00B-4725-AN	—	00D-4725-AN	00F-4725-AN	AJ0-9298
Polar C18	00A-4759-AN	00B-4759-AN	—	00D-4759-AN	00F-4759-AN	AJ0-9532
Biphenyl	00A-4622-AN	00B-4622-AN	—	00D-4622-AN	00F-4622-AN	AJ0-9209
XB-C18	00A-4496-AN	00B-4496-AN	00C-4496-AN	00D-4496-AN	00F-4496-AN	AJ0-8782
C18	00A-4462-AN	00B-4462-AN	00C-4462-AN	00D-4462-AN	00F-4462-AN	AJ0-8782
C8	00A-4497-AN	00B-4497-AN	00C-4497-AN	00D-4497-AN	00F-4497-AN	AJ0-8784
HILIC	00A-4461-AN	00B-4461-AN	00C-4461-AN	00D-4461-AN	00F-4461-AN	AJ0-8786
Phenyl-Hexyl	00A-4495-AN	00B-4495-AN	00C-4495-AN	00D-4495-AN	00F-4495-AN	AJ0-8788
F5	00A-4723-AN	00B-4723-AN	—	00D-4723-AN	00F-4723-AN	AJ0-9322
						for 2.1 mm ID

Kinetex 2.6 µm MidBore™ Columns (mm)						SecurityGuard ULTRA Cartridges‡
Phases	**30 x 3.0**	**50 x 3.0**	**75 x 3.0**	**100 x 3.0**	**150 x 3.0**	**3/pk**
EVO C18	—	00B-4725-Y0	—	00D-4725-Y0	00F-4725-Y0	AJ0-9297
Polar C18	—	00B-4759-Y0	—	00D-4759-Y0	00F-4759-Y0	AJ0-9531
Biphenyl	—	00B-4622-Y0	—	00D-4622-Y0	00F-4622-Y0	AJ0-9208
XB-C18	00A-4496-Y0	00B-4496-Y0	00C-4496-Y0	00D-4496-Y0	00F-4496-Y0	AJ0-8775
C18	00A-4462-Y0	00B-4462-Y0	00C-4462-Y0	00D-4462-Y0	00F-4462-Y0	AJ0-8775
C8	00A-4497-Y0	00B-4497-Y0	00C-4497-Y0	00D-4497-Y0	00F-4497-Y0	AJ0-8777
HILIC	00A-4461-Y0	—	—	—	00F-4461-Y0	AJ0-8779
Phenyl-Hexyl	—	00B-4495-Y0	—	00D-4495-Y0	00F-4495-Y0	AJ0-8781
F5	—	00B-4723-Y0	—	00D-4723-Y0	00F-4723-Y0	AJ0-9321
						for 3.0 mm ID

Kinetex 2.6 µm Analytical Columns (mm)						SecurityGuard ULTRA Cartridges‡
Phases	**30 x 4.6**	**50 x 4.6**	**75 x 4.6**	**100 x 4.6**	**150 x 4.6**	**3/pk**
EVO C18	—	00B-4725-E0	—	00D-4725-E0	00F-4725-E0	AJ0-9296
Polar C18	—	00B-4759-E0	—	00D-4759-E0	00F-4759-E0	AJ0-9530
Biphenyl	—	00B-4622-E0	—	00D-4622-E0	00F-4622-E0	AJ0-9207
XB-C18	—	00B-4496-E0	00C-4496-E0	00D-4496-E0	00F-4496-E0	AJ0-8768
C18	00A-4462-E0	00B-4462-E0	00C-4462-E0	00D-4462-E0	00F-4462-E0	AJ0-8768
C8	—	00B-4497-E0	00C-4497-E0	00D-4497-E0	00F-4497-E0	AJ0-8770
HILIC	—	00B-4461-E0	00C-4461-E0	00D-4461-E0	00F-4461-E0	AJ0-8772
Phenyl-Hexyl	—	00B-4495-E0	00C-4495-E0	00D-4495-E0	00F-4495-E0	AJ0-8774
F5	—	00B-4723-E0	—	00D-4723-E0	00F-4723-E0	AJ0-9320
						for 4.6 mm ID

‡SecurityGuard Ultra Cartridges require holder, Part No.: AJ0-9000
*PREP SecurityGuard Cartridges require holder, Part No. AJ0-8223
**PREP SecurityGuard Cartridges require holder, Part No. AJ0-8277

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guarantee

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