More Accurate Purity Assessments of Peptide Process Purifications Using Kinetex® Core-Shell Media

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Kinetex core-shell 2.6 µm C18 columns were used to analyze fractions from a preparative purification of the synthetic peptide bivalirudin. The ultra-high efficiency of Kinetex allowed for shorter run times while achieving improved resolution and enhanced sensitivity for impurities in the fractions. The improved performance of the Kinetex core-shell column provided more accurate purity assessments compared to fully porous HPLC columns. These improvements over an existing HPLC purity analysis method make Kinetex core-shell columns an excellent choice for analyzing fractions from preparative purifications.

Introduction

Reversed phase chromatography is typically used for the purification of synthetic peptides because of its high resolving power and sample loading capacity. After developing optimized mobile phase and gradient conditions, analyte loads of up to 5 % of the column mass are used during the preparative purification of a peptide. At such high preparative loads, the UV trace from a purification HPLC run is meaningless in assessing the purity and recovery of the synthetic peptide; thus analytical HPLC analysis of individual fractions is performed to determine which fractions to include in a final pool of the purified peptide. An important requirement of analytical HPLC analysis of such fractions is to obtain good resolution between the main peptide peak and impurities. In addition, high efficiency of impurity peaks is an important aspect of any analysis column to obtain detection and the most accurate quantitation of impurity peaks.

Core-shell technology is an innovation in analytical chromatography where the geometry of porous silica particles has been optimized to increase column efficiency. The particles are engineered with a non-porous solid core surrounded by a thin porous layer bonded with a functional ligand. An illustration of the Kinetex core-shell particle is shown in **Figure 1**. By utilizing the non-porous solid core, analytes spend less time diffusing in and out of the pore resulting in less band broadening and higher efficiency versus what is obtained from fully porous media of the same particle size. Because the Kinetex C18 column uses 2.6 µm media, one can realize similar performance to fully porous sub-2 µm particle columns but at backpressures lower than 400 bar, making such columns amenable to existing HPLC systems (thus making the purchase of a new high-pressure HPLC system unnecessary).[1]

Materials and Methods

Solvents and reagents were obtained from EMD (San Diego, CA). Crude bivalirudin was kindly supplied by CS Bio (Menlo Park, CA). All chromatography was performed on an Agilent® HP® 1100 HPLC with autosampler and UV detector (Palo Alto, CA). Data was collected using ChemStation software (Agilent) and fractions were collected using a Gilson fraction collector (Middleton, WI). For preparative purifications a Luna® 10 µm *PREP* C8(2) 250 x 4.6 mm column was used; for fraction collection either a fully porous 5 µm C8 column (250 x 4.6 mm) or Kinetex core-shell 2.6 µm C18 (100 x 4.6 mm) was used (all columns from Phenomenex Inc., Torrance, CA).

For preparative purification of crude bivalirudin, 5 mL of a 7 mg/mL solution was loaded on the Luna *PREP* C8(2) column. Mobile phases used were 0.1 % trifluoroacetic acid (TFA) in water (mobile phase A) and 0.1 % TFA in acetonitrile (mobile phase B) flowing at 1 mL/min. The gradient profile was from 15 % to 35 % B in 40 minutes with a column wash at 80 % B and re-equilibration for a total run time of 55 minutes. Analyte elution was monitored by UV at 220 nm and 0.2 minute fractions were collected.

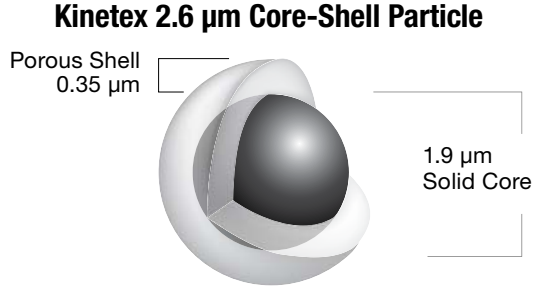
For fraction purity analysis, an aliquot of 2 µL from each fraction was injected on HPLC. A gradient from 20 % to 50 % in 30 minutes was used for the fully porous 5 µm C8 column (total run time: 45 minutes); for the Kinetex core-shell 2.6 µm C18 column a gradient from 23 % to 31 % B in 8 minutes was used (total run time: 11 minutes). In both cases column wash at 80 % B and re-equilibration were used.

Results and Discussion

Figure 1 explains the Kinetex core-shell technology and its performance advantages in general chromatography. In short, such technology allows for faster runs, higher resolution, and better sensitivity.

Figure 1.
Kinetex Core-Shell Technology

The Kinetex core-shell technology is composed of a nearly monodisperse 1.9 µm solid silica core and a 0.35 µm porous silica shell. The particle design results in a very stable (850 bar for 2.6 µm particle) and homogeneous column bed that significantly reduces peak dispersion via less eddy diffusion (the “A” term of the van Deemter equation). Additionally, the reduced diffusion path through 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).



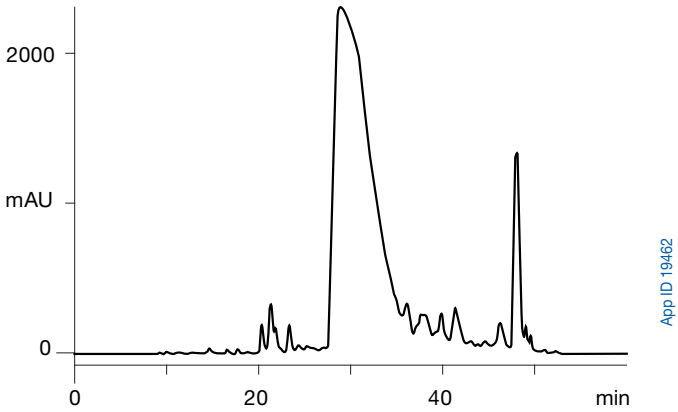
APPLICATIONS

Comparisons were performed between core-shell and fully porous media in the HPLC analysis of peptide fractions from a preparative purification. **Figure 2** is the chromatogram of preparative peptide purification runs of bivalirudin on a Luna® 10 µm *PREP* C8(2) 250 x 4.6 mm column. Different fractions across the elution profile were collected and re-analyzed using either a Kinetex® 2.6 µm C18 column or a fully porous 5 µm C8 column.

Figures 3-5 show chromatographic comparisons between the Kinetex core-shell C18 column and fully porous media. **Figure 3** compares elution of an early eluting fraction; **Figure 4** compares fraction analysis close to the center of the bivalirudin peak; and **Figure 5** compares the elution profile of later eluting impurities in the crude mixture. In every case, the Kinetex core-shell column demonstrates better resolution of the bivalirudin peak and its impurities compared to the fully porous 5 µm column despite the shorter run time for the Kinetex column. This is a direct result of the higher efficiency that core-shell media delivers.

The core-shell columns also demonstrate improved peak height resulting in greater sensitivity for low-level contaminants. Such improved sensitivity results in more accurate purity results from fraction analysis. A consequence of this improved accuracy is typically lower purity values for most fractions. A summary of fraction purity estimates for the core-shell and porous media is listed in **Table 1**. Fraction analysis using Kinetex core-shell media results in more accurate purity assessments of peptide purifications.

Figure 2.
Preparative Purification of Bivalirudin



UV elution profile for crude bivalirudin on a Luna 10 µm *PREP* C8(2) column. Approximately 35 mg (1.5 % specific load) was loaded on a 250 x 4.6 mm column and fractions were collected every 0.2 minutes and labeled according to collected retention time.

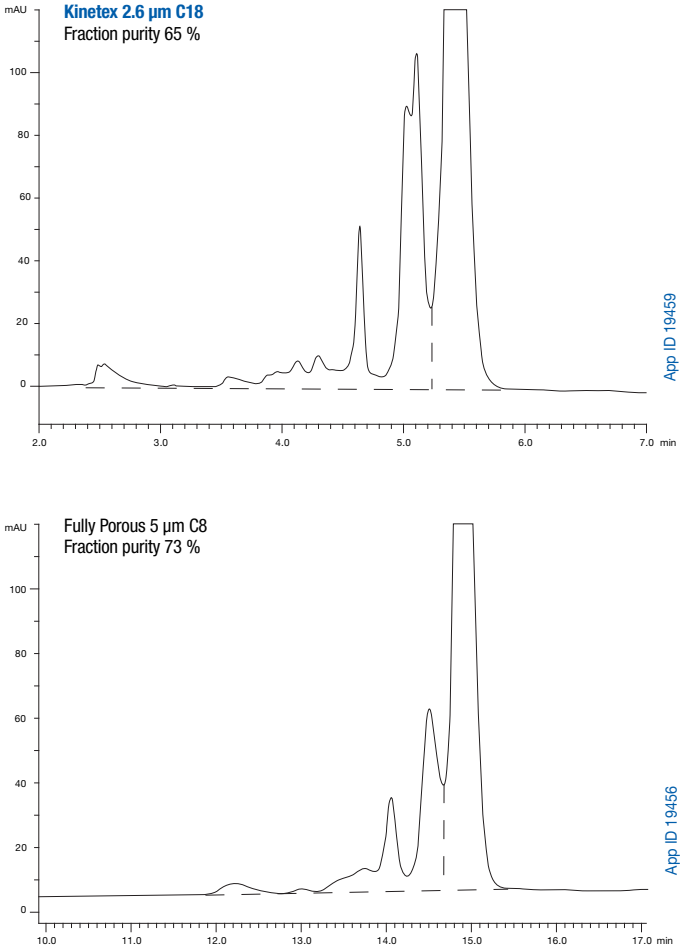
Column: Luna® 10 µm *PREP* C8(2)
Dimensions: 250 x 4.6 mm
Part No.: 00G-4323-E0
Mobile Phase: A: 0.1 % Trifluoroacetic acid in Water
B: 0.1 % Trifluoroacetic acid in Acetonitrile

Gradient:

Time (min)	% B
5	15
5.01	15
45	35
45.01	80
50	80
50.01	15
60	15

Flow Rate: 1 mL/min
Temperature: Ambient
Detection: UV @ 280 nm
Sample: Bivalirudin crude

Figure 3.
Purity Analysis of Fraction collected at 28.8 minutes

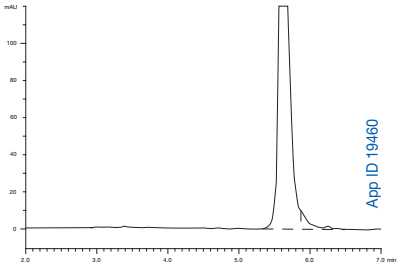


HPLC analysis of an early eluting fraction compared between a Kinetex and fully porous column. Note the narrower peaks and greater resolving power of the Kinetex core-shell column. The improved resolution gives more accurate quantitation of fraction purity.

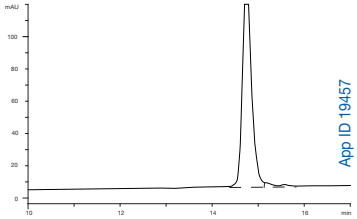
See Materials and Methods on page 1 for column conditions.

Figure 4.
Purity Analysis of Fraction collected at 32.2 minutes

Kinetex® 2.6 µm C18
Fraction purity 95 %



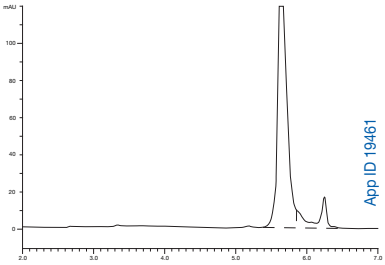
Fully Porous 5 µm C8
Fraction purity 98 %



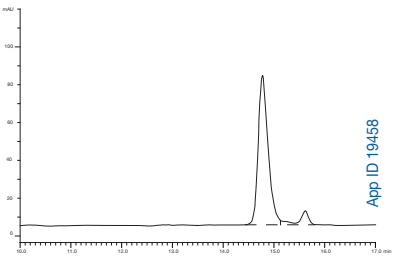
HPLC analysis of a fraction in the middle of the bivalirudin peak again compared between a Kinetex and a fully porous column. Note the increased resolution of a late-eluting peak on the Kinetex column.

Figure 5.
Purity Analysis of Fraction collected at 33.2 minutes

Kinetex 2.6 µm C18
Fraction purity 87 %



Fully Porous 5 µm C8
Fraction purity 91 %



HPLC analysis of a late eluting fraction from the preparative purification. Again note the increased efficiency of the Kinetex core-shell column. The higher efficiency results in increased recovery of impurities resulting in more accurate purity assessments.

See Materials and Methods on page 1 for column conditions.

Table 1.
Comparison of fraction purity estimates based on HPLC analysis using either Kinetex core-shell or a fully porous 5 µm column.

	Purity %	
Fraction RT Time	Fully Porous 5 µm C18	Kinetex 2.6 µm C18
28.8	73	65
29	91	87
29.2	95	93
29.4	96	96
29.6	98	96
29.8	98	95
30	99	97
30.2	98	96
30.4	97	96
30.6	96	96
30.8	96	94
31	95	93
31.2	94	96
31.4	98	96
31.6	97	94
31.8	98	95
32	98	95
32.2	98	95
32.4	98	95
32.6	97	90
32.8	95	83
33	94	83
33.2	91	87
33.4	89	81
33.6	86	72
33.8	84	72
34	80	71

Conclusion

HPLC analysis of fractions from a preparative purification is the typical method for determining peptide purity. The improved performance of Kinetex core-shell media allows for better resolution, improved sensitivity, and shorter run time for fraction analysis. An additional benefit for peptide purification is that core-shell media delivers more accurate purity assessments of isolated fractions, allowing one to make more informed decisions on which fractions to pool to deliver a desired purity and recovery.

References

1. Grittia, F.; Leonardisa, I; Shock, D.; Stevenson, P.; Shalliker, A.; Guiochon, G. Performance of columns packed with the new core-shell particles, Kinetex C18. Journal of Chromatography A, Vol 1217, Issue 10, **2010** 1589-1603.

APPLICATIONS

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Kinetex® Ordering Information

2.6 µm Analytical Columns (mm)

	30 x 4.6	50 x 4.6	75 x 4.6	100 x 4.6	150 x 4.6	SecurityGuard™ Ultra Cartridges‡ /3pk	KrudKatcher™ Ultra In-Line Filter* /3pk
XB-C18	—	00B-4496-E0	00C-4496-E0	00D-4496-E0	00F-4496-E0	AJ0-8768	AF0-8497
C18	00A-4462-E0	00B-4462-E0	00C-4462-E0	00D-4462-E0	00F-4462-E0	AJ0-8768	AF0-8497
C8	—	00B-4497-E0	00C-4497-E0	00D-4497-E0	00F-4497-E0	AJ0-8770	AF0-8497
PFP	00A-4477-E0	00B-4477-E0	00C-4477-E0	00D-4477-E0	00F-4477-E0	AJ0-8773	AF0-8497
HILIC	—	00B-4461-E0	00C-4461-E0	00D-4461-E0	00F-4461-E0	AJ0-8772	AF0-8497
							for 4.6 mm ID

‡SecurityGuard Ultra cartridges require holder, Part No.: AJ0-9000.
Check for availability in your country.

2.6 µm MidBore™ Columns (mm)

	30 x 3.0	50 x 3.0	75 x 3.0	100 x 3.0	150 x 3.0	SecurityGuard Ultra Cartridges‡ /3pk	KrudKatcher™ Ultra In-Line Filter* /3pk
XB-C18	—	00B-4496-Y0	—	00D-4496-Y0	—	AJ0-8775	AF0-8497
C18	00A-4462-Y0	00B-4462-Y0	00C-4462-Y0	00D-4462-Y0	00F-4462-Y0	AJ0-8775	AF0-8497
C8	—	00B-4497-Y0	—	00D-4497-Y0	—	AJ0-8777	AF0-8497
PFP	00A-4477-Y0	00B-4477-Y0	00C-4477-Y0	00D-4477-Y0	00F-4477-Y0	AJ0-8780	AF0-8497
HILIC	—	—	—	—	00F-4461-Y0	AJ0-8779	AF0-8497
							for 3.0 mm ID

2.6 µm Minibore Columns (mm)

	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	SecurityGuard Ultra Cartridges‡ 2.1 mm ID /3pk	KrudKatcher™ Ultra In-Line Filter* /3pk
XB-C18	00A-4496-AN	00B-4496-AN	00D-4496-AN	—	AJ0-8782	AF0-8497
C18	00A-4462-AN	00B-4462-AN	00D-4462-AN	00F-4462-AN	AJ0-8782	AF0-8497
C8	—	00B-4497-AN	00D-4497-AN	—	AJ0-8784	AF0-8497
PFP	00A-4477-AN	00B-4477-AN	00D-4477-AN	00F-4477-AN	AJ0-8787	AF0-8497
HILIC	—	00B-4461-AN	00D-4461-AN	00F-4461-AN	AJ0-8786	AF0-8497
						for 2.1 mm ID

*KrudKatcher Ultra requires $^{5}/_{16}$ in. wrench. Wrench not provided.

1.7 µm Minibore Columns (mm)

	50 x 2.1	100 x 2.1	150 x 2.1	SecurityGuard Ultra Cartridges‡ 2.1 mm ID /3pk	KrudKatcher Ultra In-Line Filter* /3pk
XB-C18	00B-4498-AN	00D-4498-AN	—	AJ0-8782	AF0-8497
C18	00B-4475-AN	00D-4475-AN	00F-4475-AN	AJ0-8782	AF0-8497
C8	00B-4499-AN	00D-4499-AN	—	AJ0-8784	AF0-8497
PFP	00B-4476-AN	00D-4476-AN	00F-4476-AN	AJ0-8787	AF0-8497
HILIC	00B-4474-AN	—	—	AJ0-8786	AF0-8497
					for 2.1 mm ID

UHPLC / HPLC Sure-Lok™ High Pressure PEEK™ Male Nut Fittings

Part No.	Description	Unit
AQ0-8503	Sure-Lok High Pressure PEEK 1-Pc Nut 10-32, For $^{1}/_{16}$ in. Tubing, 12,000 psi (827 bar)	10/pk
AQ0-8530	Sure-Lok Fitting Tightening Tool, Aluminum	ea

guarantee

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