

APPLICATIONS

A Fast and Effective Approach for the Analysis of Urinary Cortisol, Cortisone, Prednisolone and Prednisone by LC/MS/MS using StrataTM-X Solid Phase Extraction (SPE) and a Kinetex[®] core-shell Biphenyl HPLC/UHPLC Column

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The existing methods for the quantification of cortisol, cortisone, prednisolone, and prednisone are very diverse. While liquid-liquid extraction, protein precipitation, and "dilute-and-shoot" procedures offer quick and dirty methodologies, they risk increases in instrument downtime and analytical column costs. We evaluated a variety of silica-based and polymer-based SPE sorbents, each of which provides a different retention mechanism. The evaluation showed that the Strata-X polymer-based SPE sorbent, with a unique elution solvent has been found to be a robust, reproducible, and cost effective sample preparation solution for the laboratory, while providing a LLOQ of 10.0 ng/mL in human urine for all four corticosteroids.

Introduction

Cortisol is a corticosteroid hormone that stimulates anti-inflammatory and anti-stress pathways as a response to stress. Cortisone, another corticosteroid, is the inactive metabolite of cortisol. The ratio of cortisol to cortisone has clinical implications as it may be useful in diagnosis of low-renin hypertension^{3,4}.

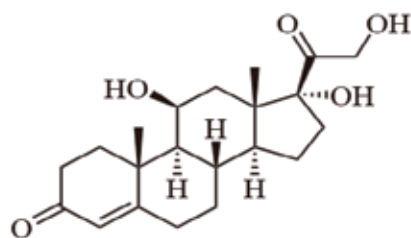
However, analysis of urinary cortisol and cortisone can be complicated if patients have been treated with prednisolone, methylprednisolone, or prednisone, as concentrations of cortisol may be falsely reported due to crossreactivity. Furthermore, prednisolone is also an isomer of cortisone, making chromatographic and spectrometric analysis difficult.

As such, a highly specific method is needed to accurately quantitate urinary cortisol and cortisone. In this study, we evaluate selected SPE sorbents, and optimize the best performing Strata-X SPE extraction methods to reach acceptable recoveries of four corticosteroids. The separation of all analytes, especially two isomers of cortisone and prednisolone, were successfully resolved by using the Kinetex core-shell Biphenyl HPLC/UHPLC column.

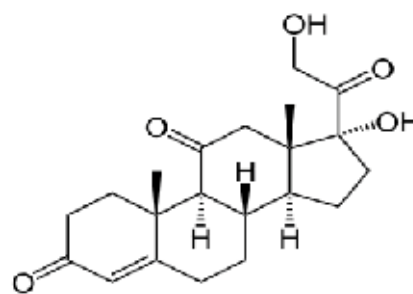
Experimental Conditions

SPE Conditions

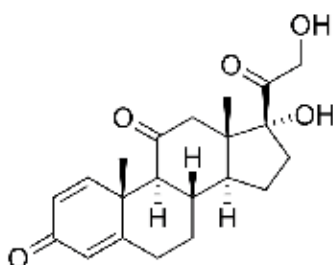
96-Well Plate: Strata-X 60 mg/well
Part No.: 8E-S100-UGB
Condition: 1 mL Methanol
Equilibrate: 1 mL Water
Load sample: 300 μ L human urine diluted in 300 μ L Water with 1 μ g/mL IS (Cortisol D4)
Wash 1: 1 mL Water
Wash 2: 1 mL 10 % Methanol in Water
Elute: 2x 500 μ L of 2 % Formic Acid in Ethyl acetate/Isopropanol (85:15)
Dry Down: To dryness under a gentle Nitrogen stream at 50 °C
Reconstitute: 100 μ L of 10 mM Ammonium acetate/10 mM Ammonium acetate in Methanol (50:50)



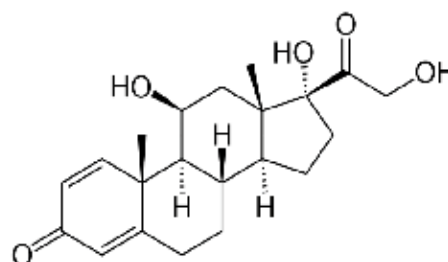
Cortisol (m.w. 362.4)



Cortisone (m.w. 360.4)



Prednisone (m.w. 358.4)



Prednisolone (m.w. 360.4)



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Experimental Conditions cont.

LC/MS/MS Conditions

LC/MS/MS was performed using a Kinetex[®] 2.6 µm core-shell Biphenyl HPLC/UHPLC column, 50 x 3.0 mm (P/N 00B-4622-Y0) on an Agilent[®] 1200SL LC system (Agilent Technologies, Palo Alto, CA, USA) with an upper pressure limit of 400 bar, equipped with a binary pump, autosampler and interfaced with an API 4000[™] triple quadrupole mass spectrometer (AB SCIEX, Framingham, MA, USA). The ionization source was electrospray ionization (ESI) analyzed in positive ion mode (**Table 1**).

Column: Kinetex 2.6 µm Biphenyl
Dimensions: 50 x 3.0 mm
Part No.: 00B-4622-Y0
Mobile Phase: A: 10 mM Ammonium acetate in Water
 B: 10 mM Ammonium acetate in Methanol

Gradient	Time (min)	% B
	0.01	40
	0.50	40
	2.00	90
	3.00	90
	3.01	40
	5.00	40

Flow Rate: 0.4 mL/min
Column Temperature: 40 °C
Injection Volume: 10 µL
Detection: MS/MS (AB SCIEX API 4000), ESI+
Instrument: Agilent[®] 1200SL with binary pumps

Table 1.
Mass Transitions and Analyte Retention Times

Analyte	Q1 Mass (Da)	Q3 Mass (Da)	Analyte Retention Time (min)
Cortisone 1	361	163	3.36
Cortisone 2	361	121	3.36
Cortisol 1	363	121	3.31
Cortisol 2	363	309	3.31
Prednisolone 1	361	147	3.19
Prednisolone 2	361	173	3.19
Prednisone 1	359	147	3.23
Prednisone 2	359	237	3.23
Cortisol-D4 1	367	121	3.22
Cortisol-D4 2	367	331	3.22

Figure 1.
Recovery of Cortisone and Cortisol using Various SPE Sorbents

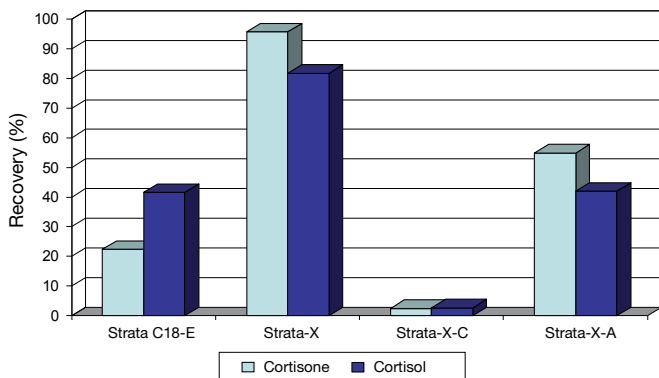
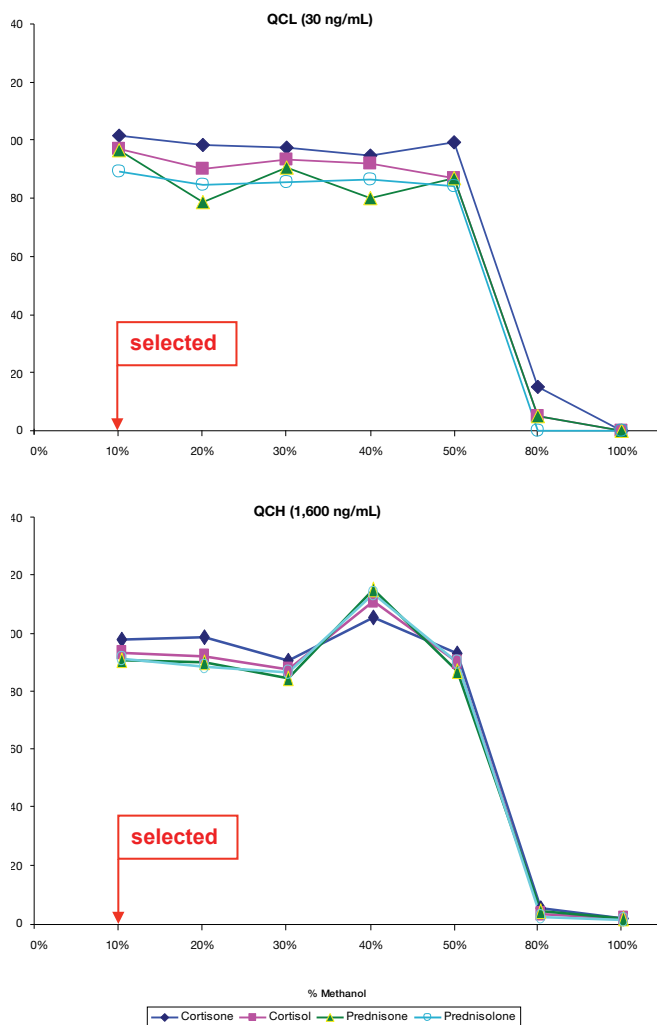


Figure 2.
Wash Solvent Optimization



Experimental Conditions cont.

Figure 3.
Recovery using Strata-X Across Low (QCL, 30 ng/mL) and High (QCH, 1600 ng/mL) QC Concentrations

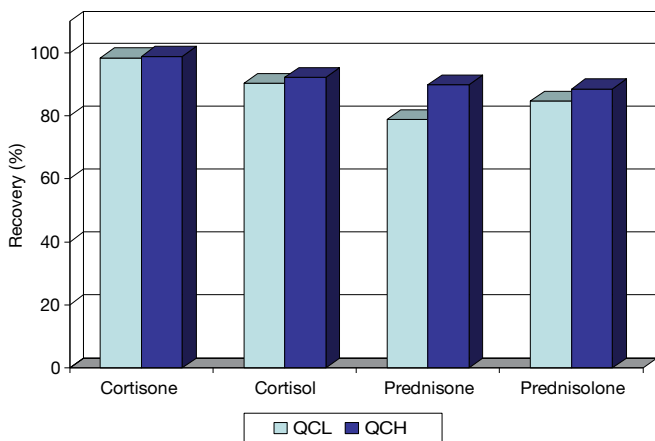


Figure 4.
Linear Regression of Cortisol

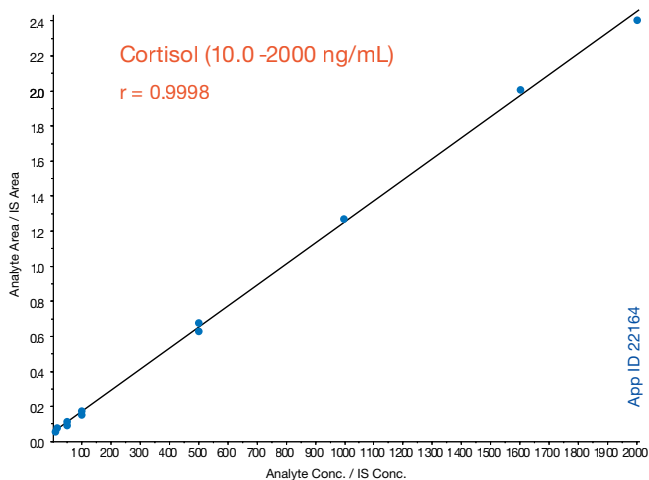


Table 2.
Accuracy and Precision

	LLOQ	QCL	QCM	QCH
Nominal Conc. (ng/mL)	10	30	500	1600
Cortisone				
Mean Conc. Fund (ng/mL)	10.1	30.4	527	1678
STDV	0.649	2.32	8.18	50.8
CV%	6.40	7.64	1.55	3.02
Accuracy (%)	101	101	106	105
Cortisol				
Mean Conc. Fund (ng/mL)	10.5	31.3	518	1660
STDV	0.399	2.41	6.65	17.9
CV%	3.82	7.71	1.28	1.08
Accuracy (%)	105	104	104	104
Prednisolone				
Mean Conc. Fund (ng/mL)	10.8	33.1	537	1587
STDV	0.973	3.49	14.9	66.5
CV%	8.99	10.5	2.78	4.19
Accuracy (%)	108	110	107	99.3
Prednisone				
Mean Conc. Fund (ng/mL)	10.2	31.1	540	1623
STDV	0.816	1.97	14.1	45.0
CV%	7.97	6.31	2.63	2.77
Accuracy (%)	102	104	108	101

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Figure 5.
Cortisol, Cortisone, Prednisolone, and Prednisone at LLOQ (10 ng/mL)

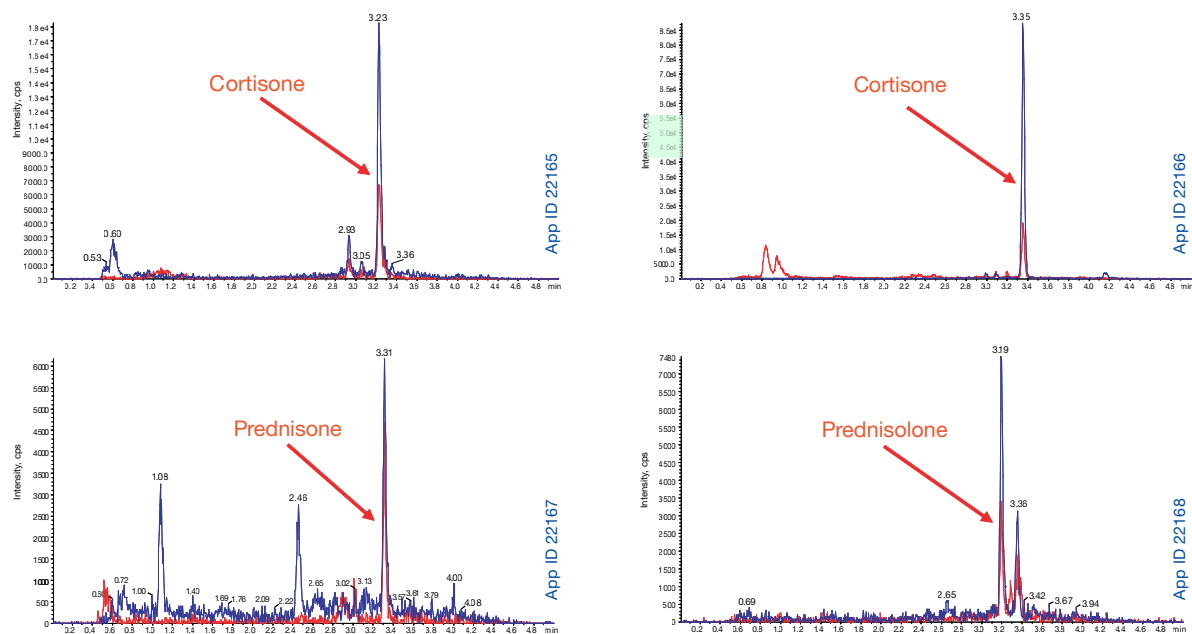


Figure 6.
Separation of Corticosteroids (1600 ng/mL)

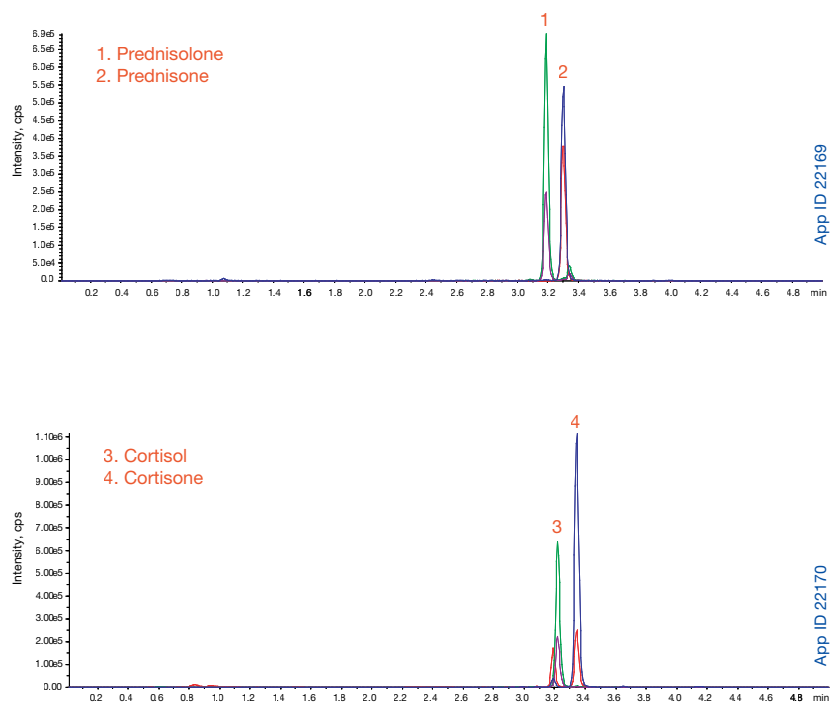
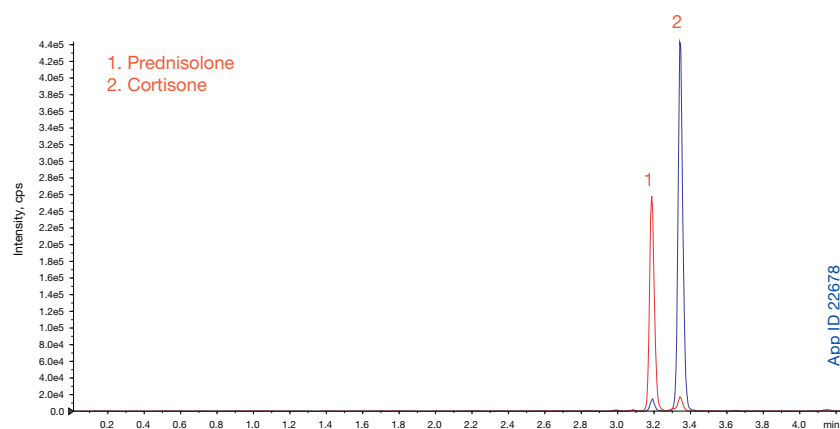


Figure 7.
Resolution of Isobaric Compounds, Prednisolone and Cortisone



Results and Discussion

The selected SPE media was compared based on manufacturer recommended procedures for each sorbent. StrataTM-X provided the best recovery (**Figure 1**) while low recoveries of analytes were observed using manufacturer recommended procedures for the other sorbents. Optimization of the second strong wash solvent was performed to maximize recovery using Strata-X. 10% Methanol in water was found to have better recovery across all the concentrations and analytes (**Figure 2**). Recoveries of four corticosteroids were acceptable at both high and low concentration QC's (**Figure 3**).

Assay linearity of all analytes was acceptable from 10.0 – 2,000 ng/mL. A representative calibration curve for cortisol is shown in **Figure 4**, resulting in a correlation coefficient of 0.9998. Accuracy and precision of 4 level QCs for all 4 compounds was between 99.3-110 % with CV% at 1.08-10.55 %, respectively (**Table 2**).

The Kinetex[®] core-shell Biphenyl HPLC/UHPLC column offered excellent sensitivity of all 4 compounds at LLOQ (10 ng/mL) (**Figure 5**) as well as separation of the two pairs of cortisone/cortisol and prednisone/prednisolone (**Figure 6**). In addition to excellent sensitivity and separation, the Kinetex core-shell Biphenyl HPLC/UHPLC column achieved separation of cortisone and prednisolone which are isobaric compounds (**Figure 7**).

Conclusions

A fast, robust LC/MS/MS method was developed for the extraction and quantitation of cortisone, cortisol, prednisone, and prednisolone from urine using Phenomenex Strata-X 96-well plates. Using a unique elution solvent, analyte recoveries were maximized. After extraction, a Kinetex core-shell Biphenyl HPLC/UHPLC column offered optimal separation and sensitivity along with baseline resolution between the isobaric compounds cortisone and prednisolone. The assay achieved an LLOQ for all four corticosteroids from urine of 10 ng/mL, proving that the method was sensitive enough to detect low levels of urinary corticosteroids.

References



1. Hoehn K, Marieb EN (2010). *Human Anatomy & Physiology*. San Francisco: Benjamin Cummings. ISBN 0-321-60261-7.
2. Mune T et al. (1995) Human hypertension caused by mutations in the kidney isozyme of 11 beta-hydroxysteroid dehydrogenase. *Nat Genet* 10: 394-399
3. Turner, S. T. *Monogenic forms of low-renin hypertension*. *Nature Clinical Practice Nephrology*, 624-630.



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Ordering Information

Strata™-X Solid Phase Extraction (SPE)

Format	Sorbent Mass	Part Number	Unit
Tab-Less Tube			
	30 mg	8L-S100-TAK	1 mL (100/box)
	60 mg	8L-S100-UBJ	3 mL (50/box)
Tube			
	30 mg	8B-S100-TAK	1 mL (100/box)
	30 mg	8B-S100-TBJ	3 mL (50/box)
	60 mg	8B-S100-UBJ	3 mL (50/box)
	100 mg	8B-S100-EBJ	3 mL (50/box)
	100 mg	8B-S100-ECH	6 mL (30/box)
	200 mg	8B-S100-FBJ	3 mL (50/box)
	200 mg	8B-S100-FCH	6 mL (30/box)
	500 mg	8B-S100-HBJ	3 mL (50/box)
	500 mg	8B-S100-HCH	6 mL (30/box)
96-Well Plate			
	10 mg	8E-S100-AGB	2 Plates/Box
	30 mg	8E-S100-TGB	2 Plates/Box
	60 mg	8E-S100-UGB	2 Plates/Box

On-line Extraction Cartridge

Description	Part No.	Unit/Box
Strata-X on-line extraction cartridge, 20 x 2.0 mm	00M-S033-B0-CB	ea
Cartridge holder, 20 mm	CH0-5845	ea

Accessories

Collection Plates (deep well, polypropylene)

AHO-7192	96-Well Collection Plate 350 µL/well	50/pk
AHO-7193	96-Well Collection Plate 1 mL/well	50/pk
AHO-7194	96-Well Collection Plate 2 mL/well	50/pk
AHO-8635	96-Well Collection Plate, 2 mL Square/Round-Conical	50/pk
AHO-8636	96-Well Collection Plate, 2 mL Round/Round, 8 mm	50/pk
AHO-7279	96-Well Collection Plate, 1 mL/well Round, 7 mm	50/pk

Sealing Mats

AHO-8597	Sealing Mats, Pierceable, 96-Square Well, Silicone	50/pk
AHO-8598	Sealing Mats, Pre-Slit, 96-Square Well, Silicone	50/pk
AHO-8631	Sealing Mats, Pierceable, 96-Round Well 7 mm, Silicone	50/pk
AHO-8632	Sealing Mats, Pre-Slit, 96-Round Well 7 mm, Silicone	50/pk
AHO-8633	Sealing Mats, Pierceable, 96-Round Well 8 mm, Silicone	50/pk
AHO-8634	Sealing Mats, Pre-Slit, 96-Round Well 8 mm, Silicone	50/pk
AHO-7362	Sealing Tape Pad	10/pk

Vacuum Manifolds

AHO-6023*	SPE 12-Position Vacuum Manifold Set, for tubes	ea
AHO-6024*	SPE 24-Position Vacuum Manifold Set, for tubes	ea
AHO-8950	96-Well Plate Manifold, Universal with Vacuum Gauge	ea

* Manifolds include: Vacuum-tight glass chamber, vacuum gauge assembly, polypropylene lid with gasket, male and female luers and yellow end plugs, stopcock valves, collection rack assemblies, polypropylene needles, lid support legs. Waste container included with 12-positive manifold.

Ordering Information Cont.

Kinetex[®] Core-Shell HPLC/UHPLC Columns

5 μ m Minibore Columns (mm)				SecurityGuard [™] ULTRA Cartridges [†]	5 μ m MidBore [™] Columns (mm)				SecurityGuard [™] ULTRA Cartridges [†]
Phase	50 x 2.1	100 x 2.1	3/pk		Phase	50 x 3.0	100 x 3.0	3/pk	
Biphenyl	00B-4627-AN	00D-4627-AN	AJ0-9209		Biphenyl	00B-4627-Y0	00D-4627-Y0	AJ0-9208	
				for 2.1 mm ID					for 3.0 mm ID

5 μ m Analytical Columns (mm)					SecurityGuard [™] ULTRA Cartridges [†]
Phase	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	3/pk
Biphenyl	00B-4627-E0	00D-4627-E0	00F-4627-E0	00G-4627-E0	AJ0-9207
					for 4.6 mm ID

2.6 μ m Minibore Columns (mm)					SecurityGuard [™] ULTRA Cartridges [†]
Phase	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
Biphenyl	00A-4622-AN	00B-4622-AN	00D-4622-AN	00F-4622-AN	AJ0-9209
					for 2.1 mm ID

2.6 μ m MidBore Columns (mm)				SecurityGuard [™] ULTRA Cartridges [†]
Phase	50 x 3.0	100 x 3.0	150 x 3.0	3/pk
Biphenyl	00B-4622-Y0	00D-4622-Y0	00F-4622-Y0	AJ0-9208
				for 3.0 mm ID

2.6 μ m Analytical Columns (mm)				SecurityGuard [™] ULTRA Cartridges [†]
Phase	50 x 4.6	100 x 4.6	150 x 4.6	3/pk
Biphenyl	00B-4622-E0	00D-4622-E0	00F-4622-E0	AJ0-9207
				for 4.6 mm ID

1.7 μ m Minibore Columns (mm)				SecurityGuard [™] ULTRA Cartridges [†]
Phase	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
Biphenyl	00B-4628-AN	00D-4628-AN	00F-4628-AN	AJ0-9209
				for 2.1 mm ID

[†] SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000



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