Rapid LC/MS/MS Analysis of Digoxin and Digitoxin in Plasma using Strata™-X SPE Cartridges and Kinetex® 2.6 µm C8 Core-Shell HPLC/UHPLC Columns

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A LC/MS/MS method has been developed for the rapid analysis of digoxin and digitoxin in plasma effective over a concentration range of 0.25 to 10 ng/mL, which covers the commonly accepted therapeutic levels for patient samples. The method described here uses Strata-X for sample clean up and concentration via solid phase extraction and a Kinetex C8 core-shell column for fast and sensitive LC/MS/MS analysis.

Introduction

Digoxin and digitoxin are cardiac glycosides that were originally extracted from the foxglove plant, *Digitalis lanata*. These two glycosides have profound effects on heart activity and are thought to function by altering the mechanisms that regulate the cardiac action potential. Functionally, the net effect of digoxin or digitoxin administration is a decrease in heart rate. As such, they may be used to treat conditions such as atrial fibrillation and atrial flutter. Interestingly, although both digoxin and digitoxin have a similar effect on cardiac activity, digitoxin is eliminated from the body via the liver, whereas digoxin is eliminated via renal activity. Therefore, digitoxin may be prescribed to patients with compromised renal function.

Controlling the dosage of digoxin and digitoxin is very important because they have a very narrow therapeutic index meaning that there is only a small difference in dosage between an effective and a toxic dose. In fact, there have been many deaths reported due to accidental overdose of these therapeutic drugs. Therefore, monitoring patient plasma levels for these compounds is of vital importance. The accepted therapeutic levels in serum for digoxin range from 0.8-2.0 ng/mL and adverse affects are more likely to be seen when plasma levels exceed 2.0 ng/mL.

We describe here a simple and accurate method for the analysis of digoxin and digitoxin in plasma which utilizes solid phase extraction using Strata-X SPE tubes followed by rapid LC/MS/MS analysis using a Kinetex core-shell C8 column. In comparison to methods that use liquid:liquid extraction, the proposed SPE method has the additional benefit of reduced waste and the ability to analyze many samples concurrently.

Materials and Methods

Reagents and Chemicals

All reagents and solvents were HPLC or analytical grade. HPLC Grade acetonitrile was purchased from Honeywell, Burdick & Jackson (Muskegon, MI); Milli-Q water was used to prepare the buffer solutions used for SPE. Digoxin, digitoxin and oleandrin (internal standard) were purchased from Sigma-Aldrich (Part Numbers D6770, 851736 and 09640, respectively).

The 10 mM ammonium acetate solutions were prepared by weighing out 0.7708 g of ammonium acetate and dissolving it in 1.0 L of water and 1.0 L of methanol.

The internal standard (IS) working solution (40 ng/mL) was prepared by dissolving 2.5 mg of Oleandrin in 2.5 mL of methanol to make a 1 mg/mL solution. The 1 mg/mL IS solution was then diluted to 40 ng/mL in 50:50 methanol/water. A 1 mg/mL digoxin and digitoxin stock solution was prepared by dissolving 2.5 mg of digoxin and digitoxin in 2.5 mL of methanol. From the 1 mg/mL stock solution a 100 ng/mL solution was made by diluting in 50:50 methanol/water. From the 100 ng/mL digoxin and digitoxin solution the calibration standards were prepared by serial dilution, yielding calibration standards at 100, 50, 25, 10, 5 and 2.5 ng/mL.

The calibration curve was generated by spiking 100 µL of each calibration standard into 900 µL of blank plasma, yielding a 1.0 mL initial sample volume. Each spiked plasma standard was prepared for analysis following the sample preparation, SPE and LC/MS/MS procedure outlined below.

Two high (7.5 ng/mL) and two low (0.75 ng/mL) QC sample solutions were prepared in plasma. Each QC sample was then prepared for LC/MS/MS analysis using the same sample preparation and SPE procedure used for the calibration curve. Each of the two replicates was then analyzed in duplicate resulting in four data points for each QC sample concentration.

Equipment and Materials

Agilent® 1200 Series HPLC (Agilent Technologies Inc., Santa Clara, CA, USA) was interfaced with API 4000™ MS/MS with ESI TurboIonSpray® (AB SCIEX™, Foster City, CA, USA) operated in positive ionization mode (ESI+).

Sample Preparation

The plasma samples were prepared as follows:

1. 1.0 mL of plasma sample was spiked with 10 µL of 40 ng/mL oleandrin internal standard and transferred to a small test tube.
2. The sample was diluted with 2 mL of D.I. water; sample ready for SPE.

Solid Phase Extraction

The prepared plasma sample is cleaned up and concentrated using SPE.

Cartridge:	Strata™-X 30 mg/3 mL
Part No.:	8B-S100-TBJ
Condition:	2 mL Methanol (1-2 mL/min)
Equilibrate:	2 mL of 10 mM Ammonium acetate in water Note: Do not let sorbent run dry
Load:	3 mL of previously prepared plasma sample (1-2 drops/sec)
Wash:	1 mL of 50:50 Methanol/10 mM ammonium acetate in water
Dry:	>10" Hg for 5-10 minutes to remove residual water
Elute:	2 mL methanol (ca. 1 drop/sec)
Drydown:	Nitrogen gas at 50 ºC
Reconstitute:	100 µL of 50:50 10 mM ammonium acetate in water/10 mM ammonium acetate in methanol, sample is ready for analysis. Note: After reconstituting transfer sample to an autosampler vial with an insert (e.g. Phenomenex, Part Number AH0-4604) to ensure the sample will be injected accurately.

APPLICATIONS

LC/MS/MS

Column: Kinetex® 2.6 µm C8 100 Å
Dimensions: 50 x 2.1 mm
Part No.: 00B-4497-AN
Mobile Phase: A: 10 mM Ammonium acetate in water
B: 10 mM Ammonium acetate in methanol

Gradient:

Time (sec)	A%	B %
0.00	50	50
2.50	0	100
2.51	50	50
5.00	50	50

Flow Rate: 0.4 mL/min
Inj. Volume: 40 µL
Temperature: 30 °C (column)
MS/MS Detection: API 4000™ MS/MS, ESI positive (ESI+)

MS/MS Conditions	
Ionization	ESI
Polarity	Positive
Scan Type	MRM
Neb	60
Drying gas	40
Collision Gas (CAD)	3
Temperature (TEM)	350
Curtain Gas (CUR)	12
IS	5500
Entrance Potential (EP)	10

Peak No.	Analyte	Retention Time (min)	Q1	Q3	Time (msec)	DP	CE	CXP
1	Digoxin	1.90	798.4	651.4	100	61	19	16
2	Oleandrin (IS)	2.35	577.2	373.2	100	40	19	12
3	Digitoxin	2.75	782.4	635.4	100	61	17	17

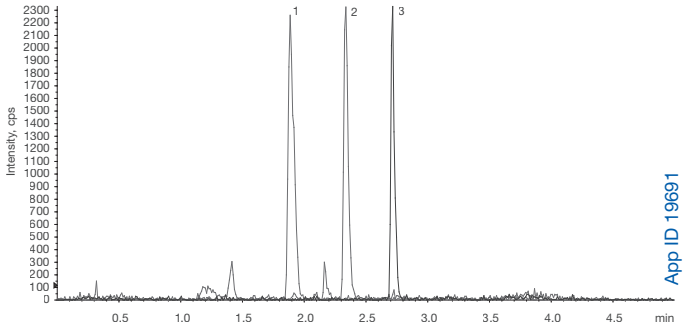
Results and Discussion

The use of the Kinetex® C8 core-shell technology column allowed for very fast elution of digoxin and digitoxin at 1.9 and 2.75 minutes, respectively (**Figure 1**). In ESI positive mode, digoxin and digitoxin were detected by monitoring the 798.4/651.4 and 782.4/635.4 mass transitions, respectively. Peak areas for both analytes were normalized using oleandrin as an internal standard, which was detected by monitoring the 577.2/373.2 mass transition.

Because of the very low analyte concentrations in patient plasma samples that need to be monitored, the only feasible detection technique is mass spectrometry. Therefore, the column technology which is utilized is of utmost importance since the analyst can use the column to improve sample throughput and sensitivity. As demonstrated, the Kinetex C8 column delivers a large amount of sensitivity while keeping analysis time to a minimum. As shown in **Figure 1**, digoxin and digitoxin are well resolved from each other and the internal standard (oleandrin); the signal-to-noise is also excellent at the lower end of the calibration range (0.25 ng/mL), allowing the typical therapeutic range to be monitored.

Solid phase extraction is often used for sample preparation of plasma samples prior to chromatographic analysis because it helps to concentrate the analytes and remove potential matrix interfering compounds present in plasma. Strata™-X is a n-vinylpyrrolidone functionalized polystyrene divinyl benzene polymer sorbent that possesses both hydrophobic and hydrophilic character, allowing for the retention of digoxin and digitoxin and removal of unretained matrix compounds. Due to the strong retention of the analytes on the Strata-X sorbent, an aggressive wash step (50:50 methanol/ammonium acetate in water) was used to remove weakly retained matrix interference. This results in a much cleaner sample extract for analysis and highlights one of the major benefits of the Strata-X sorbent.

Figure 1.
Digoxin/digitoxin (0.25 ng/mL) in plasma extracted using Strata-X
(1 = Digoxin, 2 = Oleandrin (IS), 3 = Digitoxin)



A standard calibration curve was generated over the concentration range of 0.25 ng/mL to 10 ng/mL by plotting the relative response (peak area of the analyte/peak area of the oleandrin IS) versus concentration. The standard calibration curve was linear over the calibration range with an r^2 value of 0.9999 for digoxin and an r^2 value of 0.9998 for digitoxin (**Figures 2 and 3**).

Figure 2.
Digoxin calibration curve – relative response (peak area of the analyte peak/peak area of the oleandrin IS) versus concentration (0.25 – 10 ng/mL) –extracted from plasma using Strata-X; r^2 value = 0.9999.

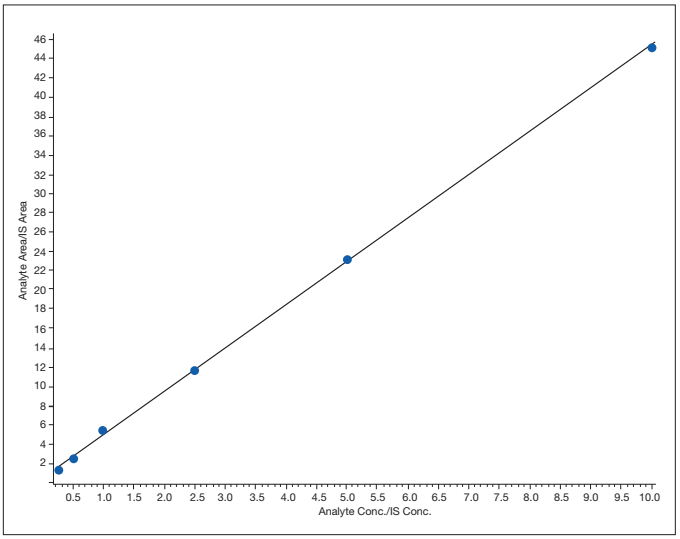
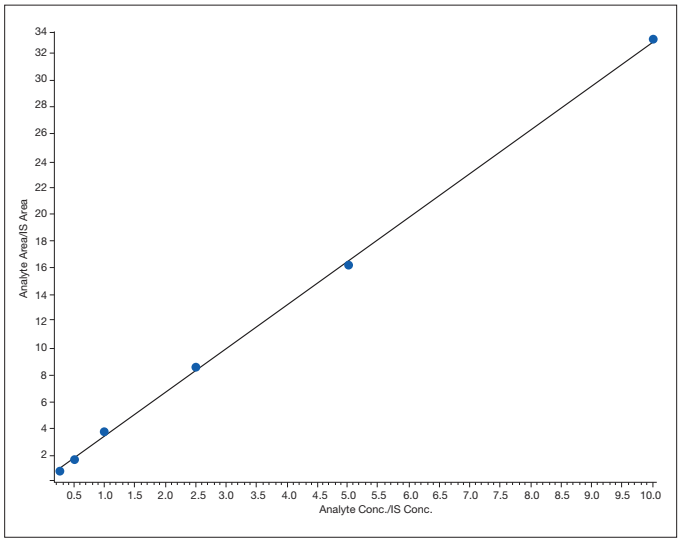


Figure 3.
Digitoxin calibration curve – relative response (peak area of the analyte peak/peak area of the oleandrin IS) versus concentration (0.25 – 10 ng/mL) –extracted from plasma using Strata-X; r^2 value = 0.9998



The extracted QC samples were made at two concentrations, one low (0.75 ng/mL) and one high (7.5 ng/mL). The QC samples were treated in the same way as the calibration curve standards, i.e. spiked with standard, diluted and then extracted with the Strata-X solid phase extraction cartridges before LC/MS/MS analysis. Excellent reproducibility was obtained for both the low and high end QC samples with RSD values < 6 % (**Table 1**).

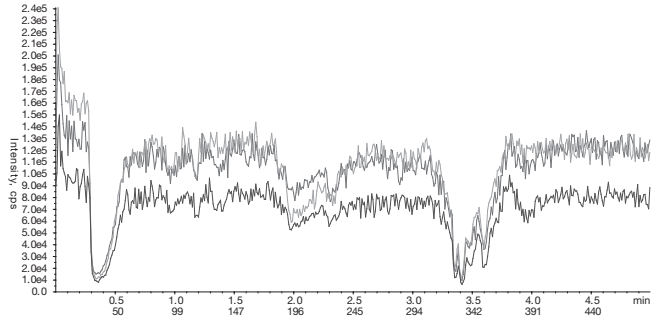
Table 1.
QC samples at low (0.75 ng/mL) and high (7.5 ng/mL) concentrations

Analyte	QC concentration (ng/mL)	RSD (%) (n=4)
Digoxin	0.75 7.5	1.3 5.8
Digitoxin	0.75 7.5	2.9 4.4

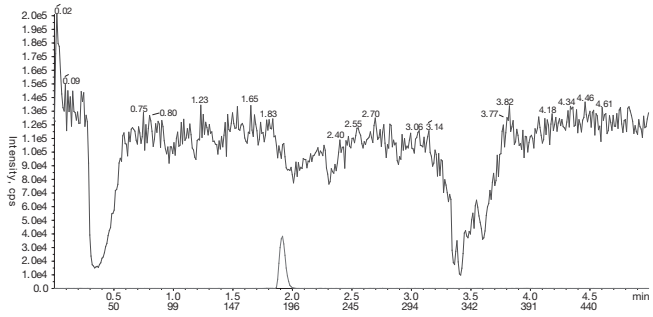
The suppression study (**Figure 4**) shows that the digitoxin peak elutes in an optimal portion of the chromatographic run (**Figure 4c**) where there is no signal suppression or enhancement. On the other hand, digoxin elutes in a portion of the run (**Figure 4b**) where some suppression is present. However, the amount of suppression is minimal and still allows for adequate signal and excellent reproducibility. This overall method provides a very linear correlation between concentration and peak response over the calibration range (0.25 – 10 ng/mL) and therapeutic dosage range for digoxin (0.8 – 2.0 ng/mL) in serum.

Figure 4.
Suppression study for Digoxin/Digitoxin (2 µg/mL) and internal standard infused through T-splitter with a blank plasma SPE extract injection.

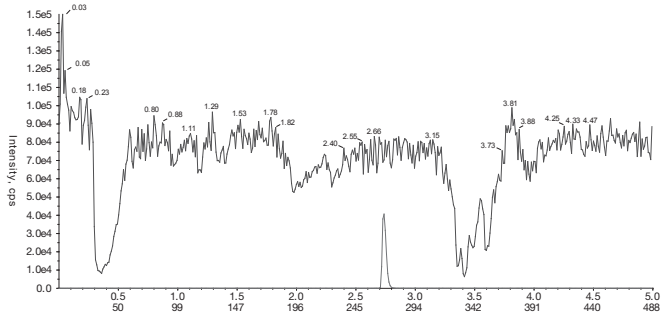
a. Blank Plasma



b. Digoxin



c. Digitoxin



Conclusion

The fast run times and and high sensitivity provided by Kinetex core-shell columns along with thorough clean-up provided by Strata-X SPE makes the proposed method very suitable for analysis of digoxin and digitoxin in a high-throughput environment.

APPLICATIONS

Ordering Information

Strata™-X

Format	Sorbent Mass	Part Number	Unit
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

Go to www.phenomenex.com to find more information on other Strata-X formats such as Teflon® tubes and large Giga™ tubes.

Kinetex® 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* 3/pk
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
PFP	00A-4477-Y0	00B-4477-Y0	00C-4477-Y0	00D-4477-Y0	00F-4477-Y0	AJ0-8780
HILIC	00A-4461-Y0	—	—	—	00F-4461-Y0	AJ0-8779
Phenyl-Hexyl	—	—	—	—	—	AJ0-8781
						for 3.0 mm ID

Kinetex 2.6 µm Minibore Columns (mm)

	30 x 2.1	50 x 2.1	75 x 2.1	100 x 2.1	150 x 2.1	SecurityGuard ULTRA Cartridges* 3/pk
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
PFP	00A-4477-AN	00B-4477-AN	00C-4477-AN	00D-4477-AN	00F-4477-AN	AJ0-8787
HILIC	00A-4461-AN	00B-4461-AN	00C-4461-AN	00D-4461-AN	00F-4461-AN	AJ0-8786
Phenyl-Hexyl	—	00B-4495-AN	—	00D-4495-AN	—	AJ0-8788
						for 2.1 mm ID

Go to www.phenomenex.com to find more information on the Kinetex 1.7 µm core-shell particle and other Kinetex column dimensions like the 4.6 mm ID.

* SecurityGuard ULTRA cartridges require holder, Part No. AJ0-9000

guarantee

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