Vitamin D Metabolite Analysis in Biological Samples Using Agilent Captiva EMR—Lipid

Application Note

Clinical Research

Authors

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Abstract

Lipids from biological samples are detrimental to the analysis of 25-hydroxyvitamin D2 and D3 metabolites. Traditional sample preparation techniques such as protein precipitation do not remove lipid interferences, and solid phase extraction requires multiple steps and method development. A rugged and reliable sample preparation method uses Agilent Captiva EMR—Lipid for 96-well plate based *in situ* protein precipitation and pass-through lipid cleanup. A novel sorbent chemistry allows selective removal of lipids, not target analytes. Intra- and interday accuracy was 90–110 %, and precision was <10 %RSD for all QC levels. Significant improvements in analytical sensitivity and robustness relative to protein precipitation were attributed to high lipid removal, and was demonstrated using postcolumn infusion, matrix effect, and phospholipid analysis experiments.

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Introduction

Vitamin D is routinely analyzed by monitoring 25-hydroxyvitamin D2 and D3 (25-OH D2 and D3) metabolites in biological samples such as plasma and serum. LC/MS/MS has gained wide acceptance for the separation and accurate quantification of vitamin D metabolites. While mass spectrometry affords high analytical sensitivity and selectivity for target analytes, biological samples contain interferants, namely phospholipids, which hinder analytical performance if not removed. This is a common issue associated with standard sample preparation protocols such as protein precipitation (PPT). Solid phase extraction can effectively remove matrix interferences, but requires multiple steps and method development.

Agilent Captiva EMR—Lipid facilitates PPT and lipid cleanup for dirty biological samples such as plasma, serum, and blood. Both a 96-well plate and 1 mL cartridges were used, and contain a solvent retention frit allowing *in-situ* PPT. Precipitated proteins are filtered as the eluent passes through the EMR—Lipid sorbent for cleanup. Unlike commercially available lipid removal products, Captiva EMR—Lipid is highly resistant to clogging and contains a novel, selective sorbent chemistry[1]. Lipids are removed through a combined mechanism of size-exclusion and hydrophobic interaction, delivering purified eluents ready for analysis.

This Application Note describes the verification of 25-OH D2 and D3 in serum by LC/MS/MS[2]. Accuracy and precision were assessed intra- and interday to ensure rugged method performance. Captiva EMR—Lipid provided effective *in situ* protein precipitation and clean extracts, demonstrated with matrix effect, postcolumn infusion, and phospholipid experiments, resulting in improved method reliability and robustness.

LC Configuration and parameters

Configuration	
Agilent 1290 Infinity II high speed pump (G7120A)	
Agilent 1290 Infinity II multisampler (G7167B)	
Agilent 1290 Infinity II multicolumn thermostat (G7116B)	
Analytical column	Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 50 mm, 2.7 µm, LC column (699775-902) Agilent InfinityLab Poroshell 120, EC-C18, 2.1 × 5 mm, 2.7 µm, guard column (821725-911)
Column temperature	30 °C
Injection volume	20 µL
Mobile phase A	H_2O + 0.1 % formic acid
Mobile phase B	Methanol + 0.1 % formic acid
Flow rate	0.5 mL/min
Gradient	Hold 0.5 minutes at 75 % B, ramp to 98 % B at 4 minutes, hold at 98 % B to 5 minutes
Post time	2 minutes

MS/MS Configuration and parameters

Configuration	
Agilent 6460 Triple Quadrupole LC/MS with Agilent Jet Stream	
MS/MS mode	Dynamic MRM
Ion mode	Positive
Drying gas temperature	250 °C
Drying gas flow	5 L/min
Nebulizer pressure	45 psi
Sheath gas temperature	325 °C
Sheath gas flow	11 L/min
Capillary voltage	5,000 V
EMV	200 V
Nozzle voltage	1,500 V

Compound	Precursor ion	Quantifier ion	Qualifier ion	Collision energy (V)	Frag (V)
25-OH D2	413.3	395.3	355.3	4	115
25-OH D2-d_3	416.3	398.3	–	4	115
25-OH D3	401.3	365.3	383.3	4	115
25-OH D3-d_3	404.3	386.3	–	4	115

Experimental

Sample preparation

- Agilent Captiva EMR—Lipid 96-well plate (5190-1000); (5190-1001 [5/pk])
- Captiva 96-deep well collection plate, 1 mL (A696001000)
- Agilent CaptiVac vacuum collar (A796) and gasket kit (A796G)
- Agilent Captiva 96-well plate cover, 10/pk (A8961007)
- DuoSeal 96-well plate seal, 10/pk (A8961008)

Accuracy and precision verification experiment

Chemicals and reagents

Human plasma, vitamin D-stripped plasma, and human serum were from Golden West Biological Inc. Human serum was used for method quantitation study while plasmas were used for matrix removal evaluations. Standards and internal standards were bought from Sigma-Aldrich as 100 µg/mL solutions. Sample preparation and LC solvents were bought from Honeywell.

Verification study

The Captiva EMR—Lipid protocol for vitamin D metabolites was tested in separate batches on three different days. Batches consisted of two double blanks, eight blanks, seven calibrators, and six quality controls (QCs) at each level (Table 1). QCs were bracketed between two sets of calibration curves, and were prepared at 10, 20, 50, 100, 250, 500, and 750 ng/mL levels for 25-OH D3 and 20, 30, 50, 100, 250, 500, and 750 ng/mL for 25-OH D2. Isotopically labeled internal standards 25-OH D3-d_3 and 25-OH D2-d_3 were spiked at 250 ng/mL.

Sample preparation

Preparation of calibrators and QCs

Calibrators and QCs were prespiked at appropriate levels and thoroughly mixed. Next, 100 µL of serum samples were transferred into a 96-well sample plate and spiked with 10 µL of internal standard. The sample plate was covered with a mat and vortexed. A 96-well plate pipette was used for subsequent transfers and aspiration mixing.

Agilent Captiva EMR—Lipid procedure

1. Add 400 µL acetonitrile with 1 % formic acid into Captiva EMR—Lipid plate wells.
2. Transfer 100 µL of serum from the sample plate into the EMR—Lipid plate wells for PPT.
3. Mix by aspirating and dispensing the solvent/serum mixture five times.
4. Wait 1 minute to allow precipitation to complete.
5. Insert CaptiVac vacuum collar between the EMR—Lipid plate and collection plate, and begin the flow with 1–2" Hg vacuum. Collect the filtrate in a 96-well collection plate.
6. Maintain 1 drop/5 sec, increasing vacuum as required.
7. Apply 10" Hg vacuum for 1 minute to complete the elution.
8. Cap the collection plate with a plate mat, and the sample is ready for analysis.

Table 1. Sample identification and QC concentrations.

Sample ID	Definition	25-OH D2 Concentration (ng/mL)	25-OH D3 Concentration (ng/mL)
LLOQ	Lower limit of quantitation	10	20
LQ	Low QC	20	30
MQ	Mid QC	250	250
HQ	High QC	500	500
ULOQ	Upper limit of quantitation	750	750

Results and Discussion

Linearity

The data were processed with MassHunter quantification software. Calibration curves gave R^2 values between 0.992 and 0.997 for 25-OH D2 and D3 over the 10–750 ng/mL range using linear regression fit and $1/x^2$ weighting (Figure S1). The accuracy of all calibrators were within ±10 % of expected values.

Accuracy and precision results

The study produced outstanding results as shown by the intraday summary in Table 2 and interday summary in Table 3. Accuracy for all QCs were 90–110 % and %RSD <10. To demonstrate that Captiva EMR—Lipid does not retain 25-OH vitamin D, the absolute recoveries of QCs were determined using a postspiked calibration curve and prespiked serum samples without internal standard correction. Absolute recoveries were between 89–106 % for all levels with %RSD <15 (Table S1). Carryover was not detected in any blank or double blank samples.

Matrix removal

In biological matrices such as plasma, proteins and phospholipids are often the most detrimental interferants. Proteins were effectively removed with *in situ* PPT using a 1:4 ratio of sample/acetonitrile with 1 % formic acid as previously described. Phospholipid removal and ion suppression were evaluated using many techniques.

Table 2. Intraday accuracy and precision from method verification of 25-OH D2 and D3 in human serum (n = 6 at each QC level)

	25-OH D2		25-OH D3	
	Accuracy (%)	%RSD	Accuracy (%)	%RSD
Day 1				
LLOQ	97.2	4.7	106.8	9.9
LQ	97.8	5.8	104.1	5.9
MQ	96.0	5.3	93.4	2.8
HQ	107.3	4.9	105.1	2.7
ULOQ	101.9	2.7	101.5	3.1
Day 2				
LLOQ	108.4	5.8	108.3	6.6
LQ	101.2	4.8	99.3	7.4
MQ	108.7	5.6	108.1	4.7
HQ	105.4	3.6	108.7	2.7
ULOQ	104.3	2.9	109.1	1.5
Day 3				
LLOQ	99.1	5.6	109.4	8.1
LQ	92.9	4.8	99.2	5.4
MQ	105.0	4.6	104.0	4.0
HQ	106.0	3.1	105.8	3.0
ULOQ	105.6	2.7	109.1	3.7

Table 3. Accuracy and precision results for interday QC samples (n = 18).

	25-OH D2		25-OH D3	
	Accuracy (%)	%RSD	Accuracy (%)	%RSD
LLOQ	101.6	5.4	108.2	8.2
LQ	97.3	5.1	100.9	6.2
MQ	103.2	5.2	101.9	3.8
HQ	106.2	3.9	106.5	2.8
ULOQ	103.9	2.8	106.6	2.8

Monitoring phospholipid removal by LC/MS/MS

Phospholipid removal analysis used MS/MS precursor ion scan for *m/z* = 184, as shown in Figure 1. The high abundance blue trace is unremoved plasma phospholipids from PPT only. The small red, green, and black traces are triplicate runs for three samples of plasma after Captiva EMR—Lipid cleanup. The matrix removal from Captiva EMR—Lipid was calculated at 99.53 % using Equation 1.

$$\text{PLR \%} = \frac{(\text{Peak Area}_{\text{Blank no cleanup}} - \text{Peak Area}_{\text{Blank Captiva cleanup}})}{(\text{Peak Area}_{\text{Blank no cleanup}} - \text{Peak Area}_{\text{Reagent blank}})} \times 100$$

Equation 1. Calculation for percent phospholipid removal (% PLR) using total peak areas.

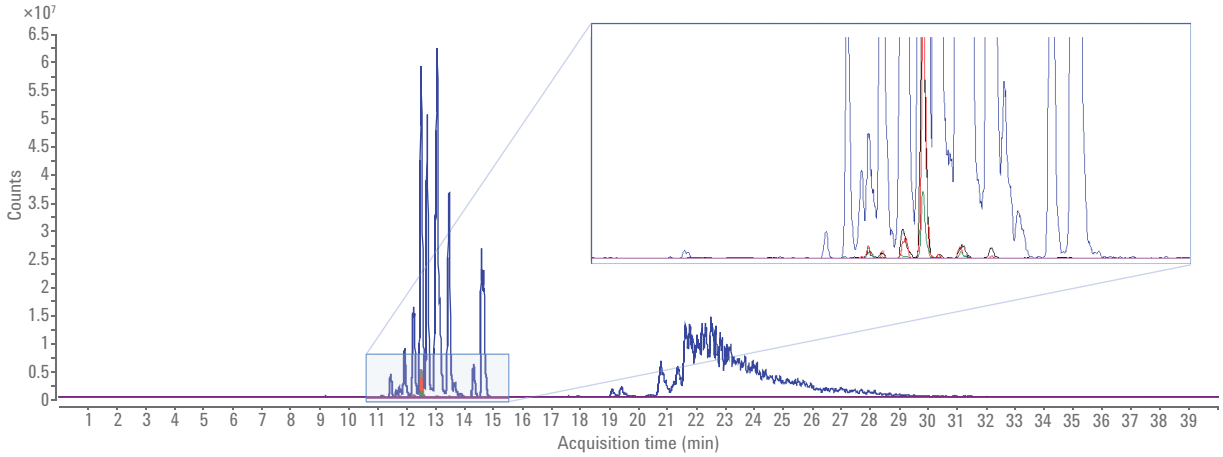

Figure 1. Phospholipid analysis product ion scan overlay of *m/z* = 184. Blue trace = PPT. Green, red, and black traces = Agilent Captiva EMR–Lipid (n = 3).

Matrix effects

Plasma samples were postspiked with 25-OH D2 and D3 at 50 ng/mL following the Captiva EMR—Lipid workflow (A) and following PPT (B) to compare response reproducibility and relative peak area. Figure 2 shows that Captiva EMR—Lipid cleanup gave highly consistent peak areas, with a %RSD < 3. The PPT samples gave analyte responses up to 80 % lower than Captiva EMR—Lipid-treated samples, and gave variable peak areas with %RSD >25.

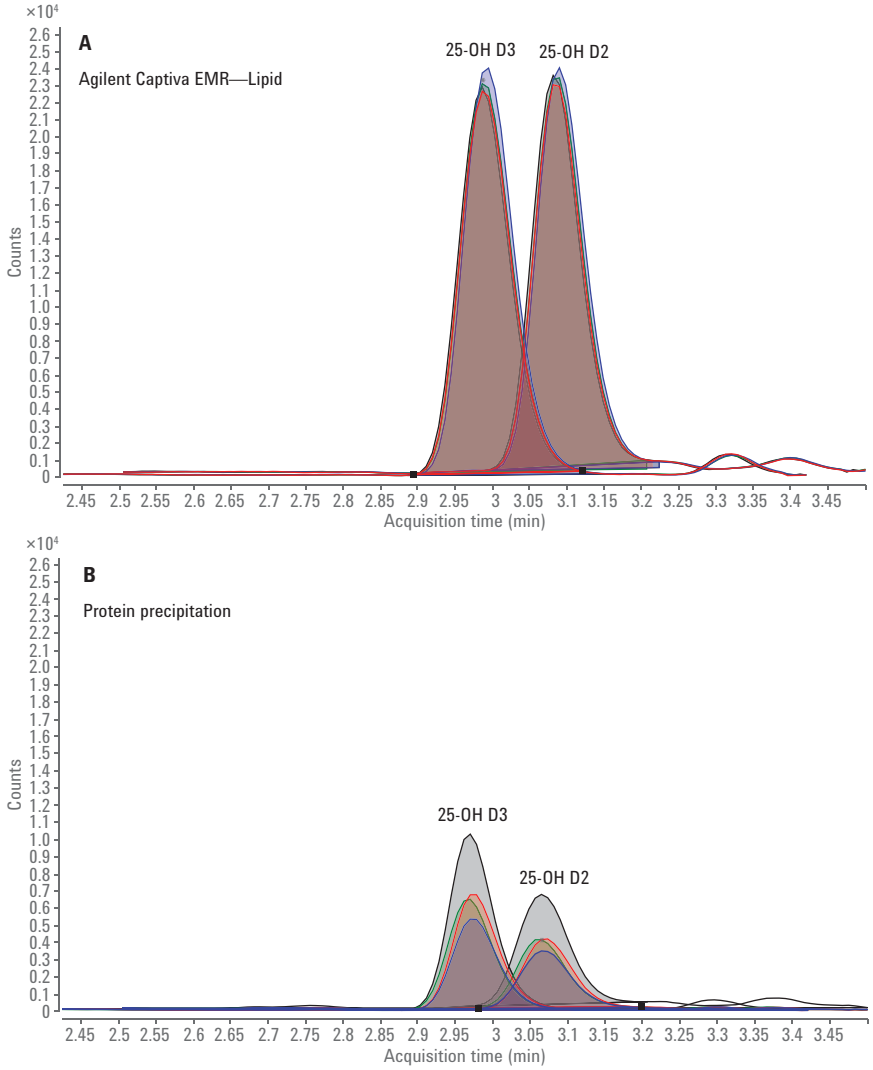

Figure 2. MRM overlay (n = 4) of 1) 25-OH D2 (*m/z* 313.3 >295.3) and 2) 25-OH D3 (*m/z* 303.3 >265.3) at 50 ng/mL in human plasma after Agilent Captiva EMR—Lipid cleanup (A) and PPT (B).

Standard postcolumn infusion

Standard postcolumn infusion was used as a qualitative method to evaluate matrix effect on target 25-OH vitamin D compounds. A 50 ng/mL solution of 25-OH D2 and D3 was pumped through a T-in after the LC column at 90 µL/h using a syringe pump with the LC method running simultaneously. Blank plasma samples were then injected producing suppression zones where matrix/lipids elute. Figure 3 shows that 25-OH D2 and D3 coelute with a suppression zone in the Figure 3B chromatogram overlay. The Figure 3A overlay shows the removal of the suppression signals with Captiva EMR—Lipid, significantly improving analyte response.

Sample preparation with Agilent Captiva EMR—Lipid

The EMR—Lipid plates were simple to use, provided efficient cleanup, and gave high analyte recovery and precision for vitamin D metabolites. Plasma QCs and calibrators were prepared in a separate sample plate to ensure thorough mixing, uniform transfer, and complete PPT. The solvent retention frit allowed retention of the crash solvent and easy flow with vacuum after PPT. Proteins were efficiently filtered without clogging, consistently giving clear eluents ready for injection. Method development showed that analytical sensitivity was increased by eluent evaporation and reconstitution, but was undesirable due to adequate sensitivity without concentration and preference for a fast, direct injection workflow. Flow was carefully controlled, allowing initiation at 1" Hg, and ramping up vacuum to 3" Hg to maintain 1 drop/5 sec. Matrix removal for all major lipid classes was consistently high for a wide variety of sample types, some of which extend beyond the scope of this work[1,3].

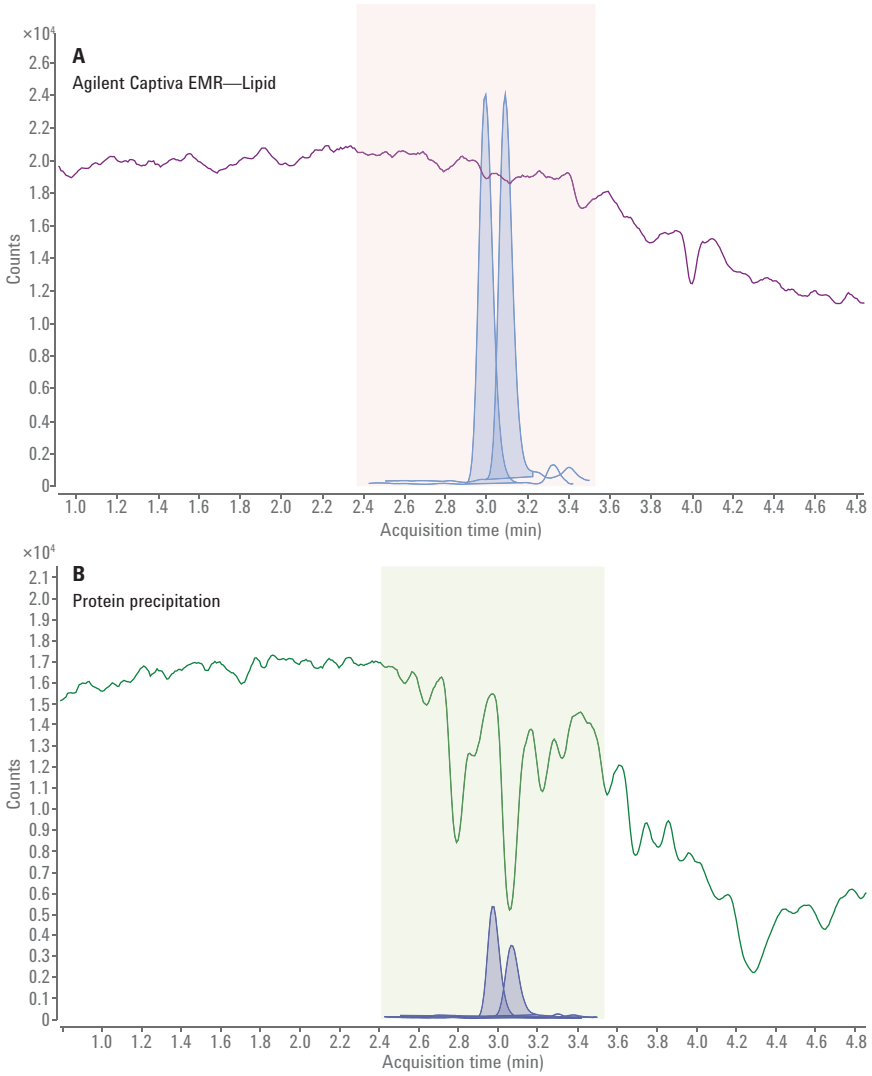

Figure 3. Chromatogram overlays of a postcolumn infusion trace at 50 ng/mL. PPT only (B). Agilent Captiva EMR—Lipid (A).

Conclusions

Excellent accuracy (90–110 %), precision (<10 %RSD), and cleanup results (>99 % phospholipid removal) were achieved using the Agilent Captiva EMR—Lipid 96-well plate for the multiday method verification of vitamin D metabolites. Proteins were effectively filtered from plasma and serum samples using *in situ* protein precipitation without clogging, while lipids were captured with the newly developed EMR—Lipid sorbent. The method is simple to perform, fast, and provides superior matrix removal ensuring maximum analytical sensitivity, minimal carryover, and high reproducibility. The sorbent selectivity for lipids allows for multiclass drug analysis[3], and future work will expand applications into other complex samples such as foods where multiclass, multiresidue analysis is highly desirable.

References

1. L. Zhao, D. Lucas, *Agilent Technologies*, publication number 5991-8006EN.

2. U.S. Department of Health and Human Services (HHS), Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM). Guidance for Industry Bioanalytical Method Validation, **2001**.

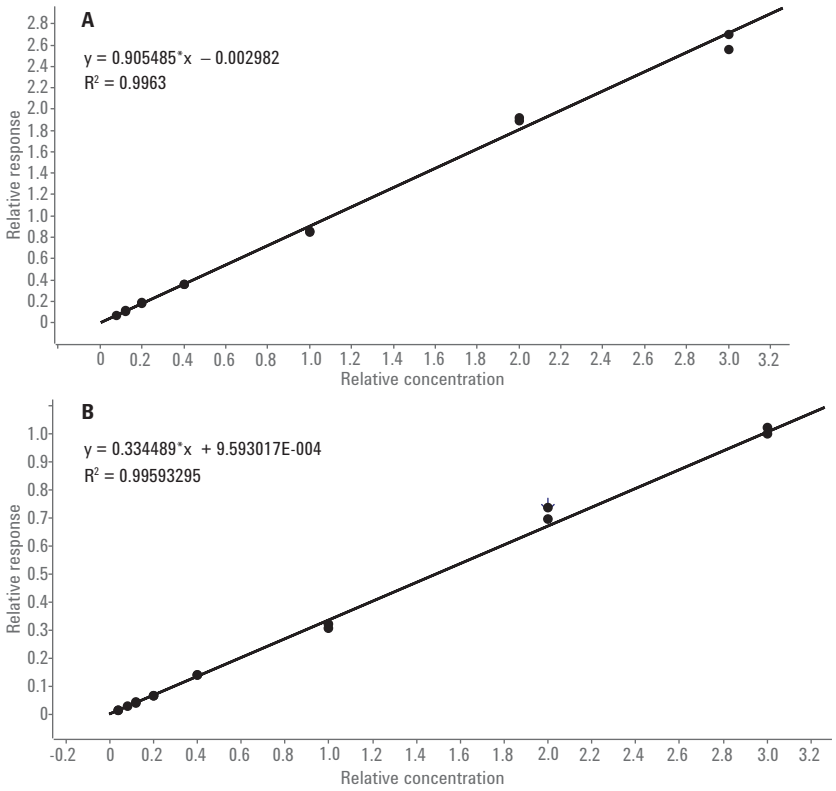

Figure S1. Selected calibration curves from 20–750 ng/mL for 25-OH D2 (A) and from 10–750 ng/mL for 25-OH D3 (B).

3. L. Zhao, D. Lucas, *Agilent Technologies*, publication number 5991-8007EN.

Table S1. Absolute recovery and % RSD values (n = 6) for 25-OH D2 and D3.

	25-OH D2		25-OH D3	
	Absolute recovery (%)	%RSD	Absolute recovery (%)	%RSD
10 ng/mL	106.4	4.8	106.3	10.2
20 ng/mL	88.8	9.5	94.2	9.2
30 ng/mL	90.8	5.0	94.7	6.3
250 ng/mL	96.9	14.6	96.6	4.2
500 ng/mL	97.1	11.5	100.1	9.5
750 ng/mL	96.9	6.4	100.3	5.0

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Published in the USA, July 11, 2017
5991-7956EN

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