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## Introduction

Improvements in LC-MS/MS sensitivity and multiple reaction monitoring (MRM) capabilities are resulting in further adoption of LC-MS/MS technology in clinical settings. One specific clinical challenge with LC-MS/MS is the potential for matrix effects that cause interferences or impact ionization efficiency. As samples vary from patient to patient it can be challenging to anticipate and detect matrix effects.

Stable isotope-labeled internal standards are frequently used to compensate for matrix effects and to increase the accuracy of quantitation. A labeled internal standard that co-elutes with the drug being monitored can offset patient specific matrix effects (co-eluting concomitant medication, etc.) that may occur at the retention time of the analyte of interest.

Complications in the use of deuterium-labeled internal standards can arise from hydrogen-deuterium scrambling in solution or in the ion source at the selected transitions. In this study, we examined deuterium-labeled hormones and other compounds of clinical significance by LC-MS/MS at select transitions. We investigated reproducibility of the scrambling ratio and influences on scrambling from different LC-MS systems (tandem quadrupole vs. quadrupole time-of-flight), concentration, solution behavior, and deuterium placement in the internal standard.

## Infusion Experiments of Clinically Significant Compounds Using QToF MS

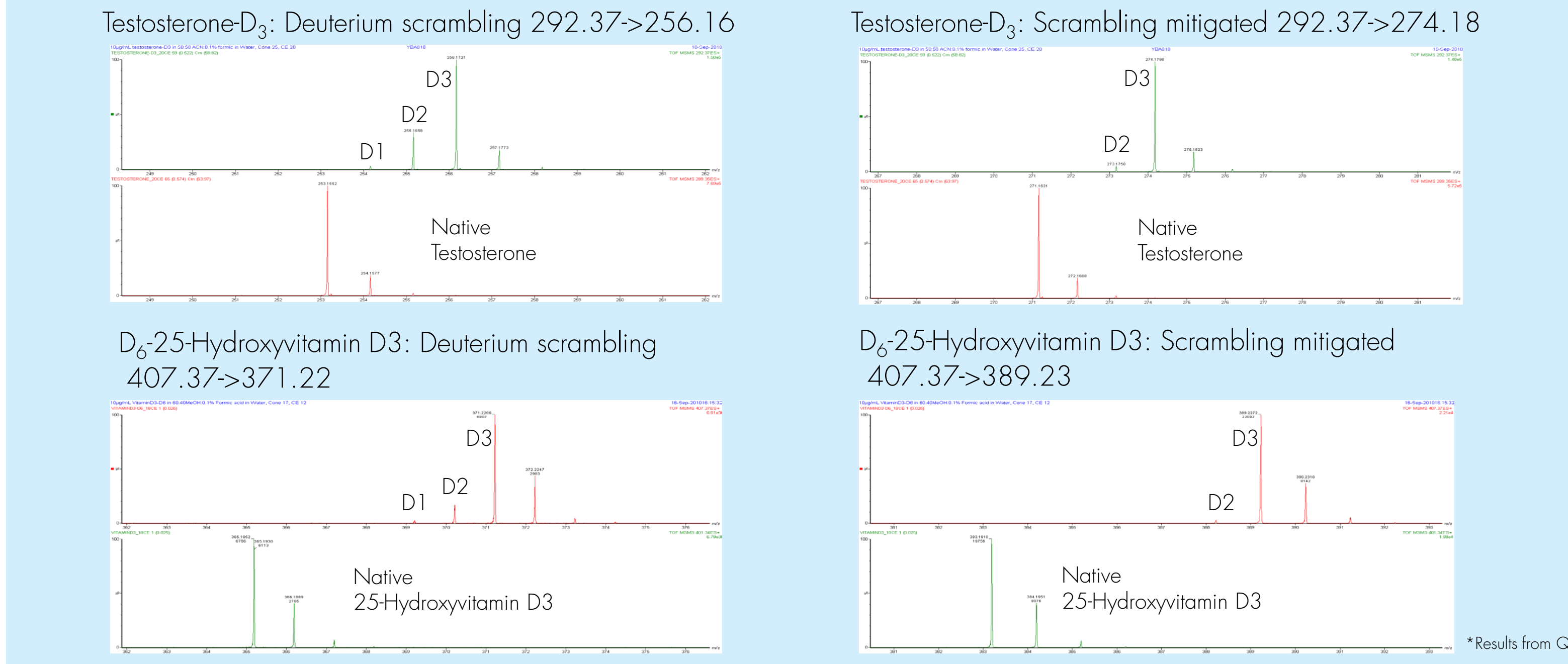
- Initial experiments were run to determine transitions and whether scrambling can occur at a selected transition.
- Instrument: Waters Xevo G2 QToF in ESI+ mode.
- Progesterone, Testosterone, Pregabalin, and Gabapentin (native and labeled) were infused at a concentration of 10 µg/mL in 50:50 ACN:0.1% formic acid in water at a flow rate of 20 µL/min.
- 25-Hydroxyvitamin D2 and D3 (native and labeled) were infused at a concentration of 10 µg/mL in 60:40 MeOH:0.1% formic acid in water at a flow rate of 20 µL/min.
- Infusion and MS parameters were optimized for signal and collision energy to give a good fragmentation pattern for each precursor ion.

Compound	Label	Major Transitions	Scrambling % D <sub>n+1</sub> /D <sub>n</sub>	Collision Energy
Testosterone	D3	292.37->256.16	33	20
		292.37->274.18	5	20
		292.37->109.05	0	20
Progesterone	D9	324.25->306.24	20	19
		324.25->288.23	22	19
		324.25->113.07	0	19
		324.25->100.07	0	19
Pregabalin	D6	166.13->148.09	0	25
		166.13->130.09	0	25
		166.13->103.08	12	25
Gabapentin	D10	182.31->164.11	0	18
		182.31->147.09	0	18
25-Hydroxyvitamin D3	D6	407.37->389.23	2	12
		407.37->371.22	12	12
25-Hydroxyvitamin D2	D6	419.60->383.40	6	10
		419.60->337.33	0	10
25-Hydroxyvitamin D2	D3	416.40->380.38	12	10
		416.40->340.35	5	10

Underlined numbers denote scrambling above 5.0%.

With initial monitoring & evaluation, transitions can be selected to minimize or eliminate deuterium scrambling in the internal standard.

## Mitigation of Scrambling by Selection of an Appropriate Transition



## Deuterium Scrambling in Clinically Significant Hormones from Multiple Reaction Monitoring (MRM) Experiments at High and Low Concentrations in Solution

- Experiments were conducted on a Waters Xevo TQ MS tandem quadrupole instrument using ESI+ mode. A Gradient system was used to elute the analytes from an ACQUITY BEH C18 2.1 x 50 mm column using water and methanol each containing 0.1% formic acid.

Hormones (Native & Labeled)	Solution	Low Concentration (ng/ml)	High Concentration (ng/ml)
Testosterone	1:1 Methanol:Water	0.5	20
Progesterone, Cortisol	1:1 Methanol:Water	10	500
Estradiol	1:1 Methanol:Water	1	500
25-Hydroxyvitamin D2/D3	60:40 Methanol:Water	30	500

- Solutions were injected and MS parameters were adjusted to give optimal signal and fragmentation for each analyte.

Hormones	Label	Major Transitions	Scrambling % D <sub>n+1</sub> /D <sub>n</sub>	Scrambling % D <sub>n+1</sub> /D <sub>n</sub>	Scrambling % D <sub>n+2</sub> /D <sub>n</sub>	Collision Energy
Testosterone		289.25->96.9	0.1	0.0	NA	30
		289.25->108.9	0.1	0.0	NA	30
		289.25->253.1	0.0	NA	0.2	20
		289.25->271.1	0.0	NA	0.0	20
Testosterone	D2	291.25->98.9	0.5	0.0	NA	30
		291.25->110.9	0.6	0.1	NA	30
		291.25->253.1	7.8	NA	0.3	20
		291.25->271.1	1.4	NA	0.0	20
Testosterone	D3	292.25->96.9	0.8	1.3	NA	30
		292.25->108.9	0.6	1.6	NA	30
		292.25->256.1	24.4	NA	1.8	20
		292.25->274.1	3.7	NA	0.1	20
Testosterone	D5	294.25->99.9	1.2	0.4	NA	30
		294.25->112.9	2.6	2.2	NA	30
		294.25->258.1	21.2	NA	2.7	20
		294.25->276.1	9.7	NA	0.3	20

Underlined numbers denote scrambling above 5.0%.

Hormones	Label	Major Transitions	Scrambling % D <sub>n+1</sub> /D <sub>n</sub>	Scrambling % D <sub>n+1</sub> /D <sub>n</sub>	Scrambling % D <sub>n+2</sub> /D <sub>n</sub>	Collision Energy
Progesterone		315.25->96.9	0.1	0.0	NA	30
		315.25->108.9	0.1	0.0	NA	30
		315.25->279.1	0.0	NA	0.3	20
		315.25->297.1	0.0	NA	0.0	20
Progesterone	D9	324.25->99.9	1.3	0.6	NA	30
		324.25->112.9	1.9	2.4	NA	30
		324.25->288.1	40.4	NA	12.4	20
		324.25->306.1	16.1	NA	0.9	20
Estradiol		*255.15->133.0	0.1	0.0	NA	25
		*255.15->159.0	0	0.2	NA	25
Estradiol	D5	*260.15->135.0	1.6	6.0	NA	25
		*260.15->161.0	1.5	8.8	NA	25
Cortisol		363.25->121.0	0.0	NA	NA	25
		363.25->309.1	0.4	NA	0.1	25
		363.25->327.1	0.5	NA	NA	25
		363.25->345.1	0.5	NA	NA	25
Cortisol	D2	365.25->123.0	48.3	NA	NA	25
		365.25->311.1	8.4	NA	0.7	25
		365.25->329.1	6.6	NA	NA	25
		365.25->347.1	7.5	NA	NA	25
Cortisol	D4	367.25->121.0	0.4	NA	NA	25
		367.25->313.1	32.1	NA	10.0	25
		367.25->331.1	18.7	NA	NA	25
		367.25->349.1	6.4	NA	NA	25

\* = Water loss used as precursor ion in Positive mode. Underlined numbers denote scrambling above 5.0%.

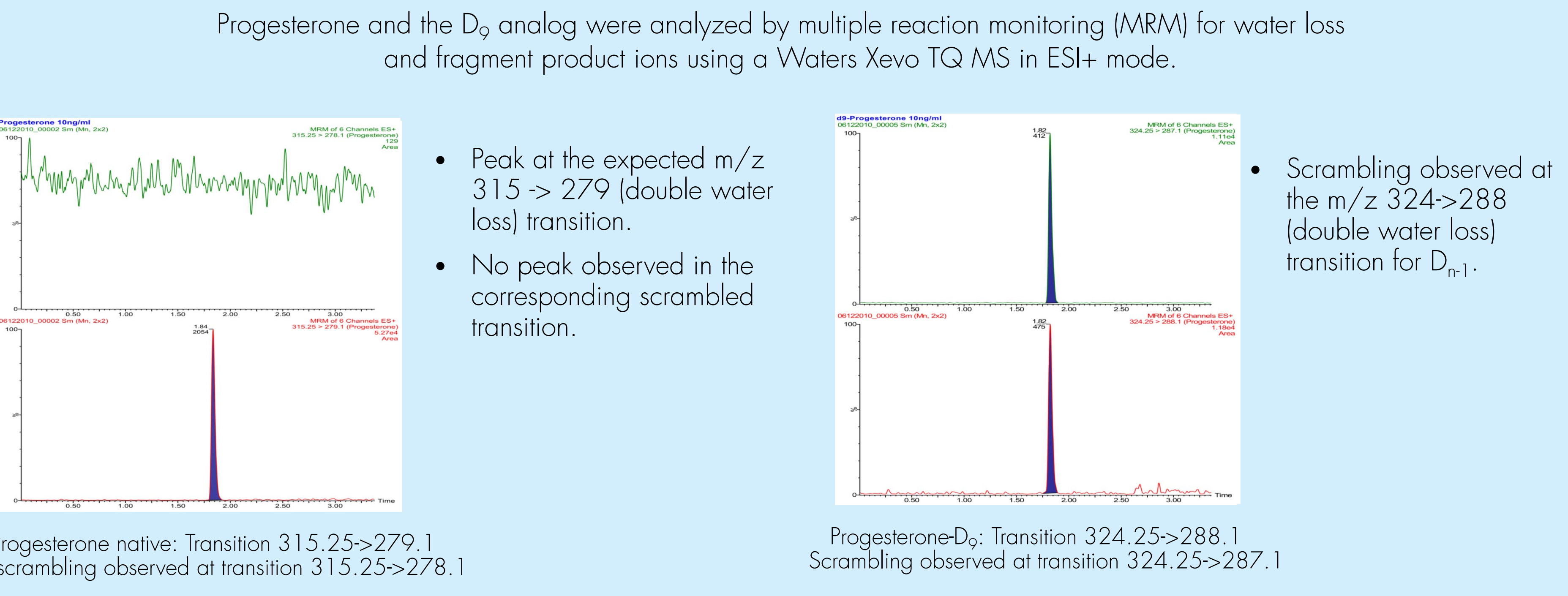
Optimal MRM Selection for Deuterium-labeled Internal Standards can be achieved in all cases except Cortisol-D<sub>2</sub>.

Hormones	Label	Major Transitions	Scrambling % D <sub>n+1</sub> /D <sub>n</sub>	Scrambling % D <sub>n+1</sub> /D <sub>n</sub>	Scrambling % D <sub>n+2</sub> /D <sub>n</sub>	Collision Energy
25-Hydroxyvitamin D2		413.35->337.25	0.0	NA	NA	15
		413.35->355.25	0.1	NA	NA	15
		413.35->377.25	0.0	NA	NA	15
		413.35->395.25	0.0	NA	NA	15
25-Hydroxyvitamin D2	D3	416.35->340.25	10.1	NA	NA	15
		416.35->358.25	0.4	NA	NA	15
		416.35->380.25	15.0	NA	NA	15
		416.35->399.25	1.7	NA	NA	15
25-Hydroxyvitamin D2	D6	419.35->337.25	0.4	NA	NA	15
		419.35->355.25	0.2	NA	NA	15
		419.35->383.25	7.6	NA	NA	15
		419.35->401.25	1.6	NA	NA	15
25-Hydroxyvitamin D3		401.35->159.0	0.2	NA	NA	30
		401.35->107.0	0.1	NA	NA	30
		401.35->365.25	0.1	NA	NA	10
		401.35->383.25	0.0	NA	NA	10
25-Hydroxyvitamin D3	D3	404.35->162.0	42.6	NA	NA	30
		404.35->110.0	52.4	NA	NA	30
		404.35->368.25	11.6	NA	NA	10
		404.35->386.25	1.0	NA	NA	10
25-Hydroxyvitamin D3	D6	407.35->159.0	0.7	NA	NA	30
		407.35->107.0	1.3	NA	NA	30
		407.35->371.25	20.2	NA	NA	10
		407.35->389.25	4.3	NA	NA	10

Underlined numbers denote scrambling above 5.0%.

Scrambling was not concentration dependant. The scrambling ratios were reproducible and consistent from high to low concentration. Deuterium placement in the internal standard can influence level of scrambling as demonstrated by the change in scrambling percent values for different deuterium analogs of cortisol and 25-hydroxyvitamin D2/D3.

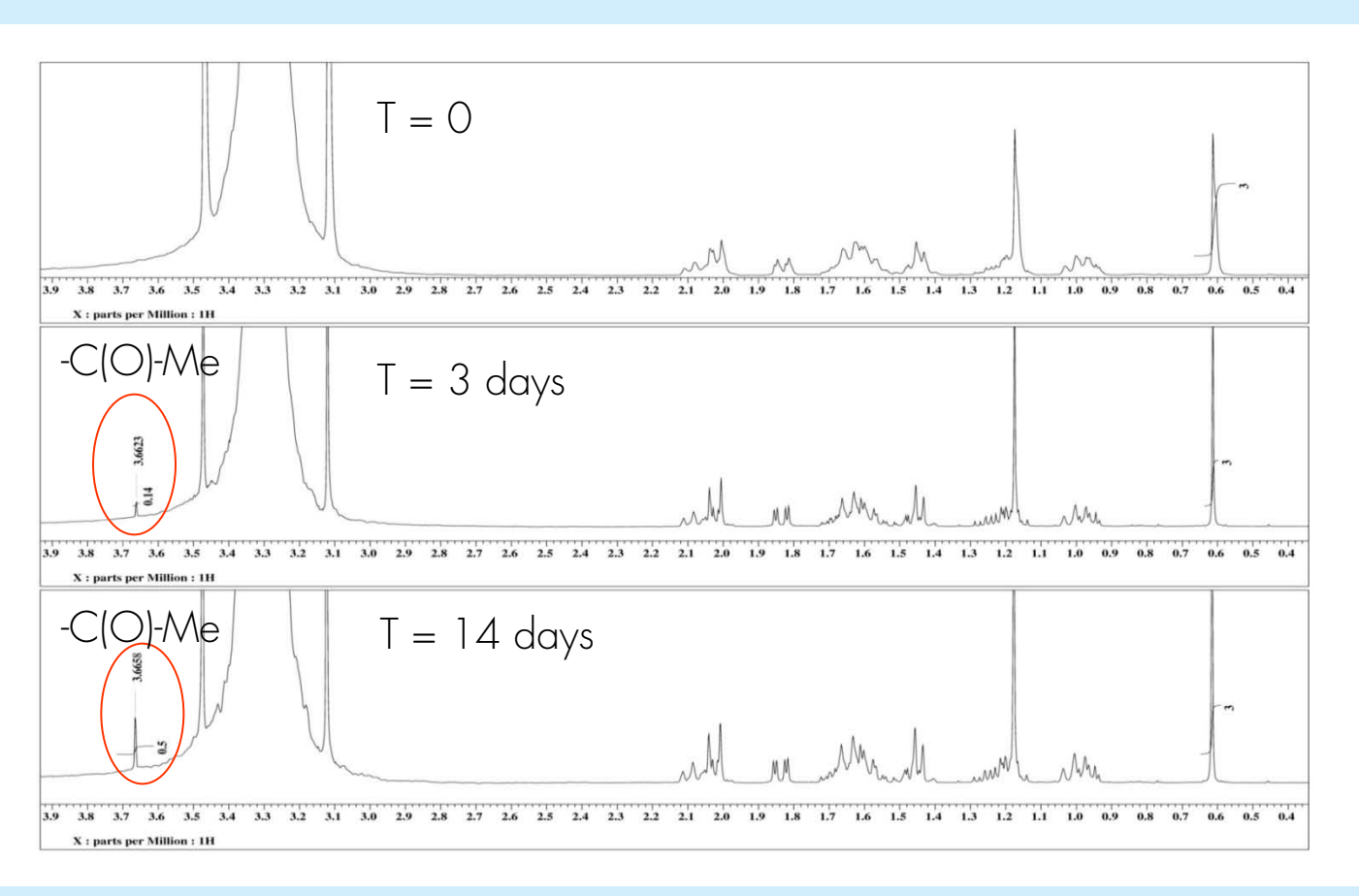
## A Representative Example of Deuterium Scrambling on Tandem Quad MS: Progesterone-D<sub>9</sub> (324->287)



Deuterium scrambling in Progesterone-D<sub>9</sub> can be mitigated by monitoring alternative MRM transitions to the water loss - for example, 324->113.

## Solution Behavior of Deuterium-Labeled Hormones by <sup>1</sup>H NMR

- Some deuterium-labeled internal standards contain deuterium at chemically exchangeable positions (example: Progesterone-D<sub>9</sub>).
- An NMR experiment was conducted to understand whether the loss of deuterium was due to exchange in solution or scrambling within the LC-MS/MS.
- Testosterone-D<sub>3</sub> (1 $\beta$ , 16 $\beta$ , 17-D<sub>3</sub>), Estradiol-D<sub>5</sub> (2 $\alpha$ , 16 $\beta$ , 17-D<sub>5</sub>), and Progesterone-D<sub>9</sub> (2,2,4,6,6,17 $\alpha$ , 21,21,21-D<sub>9</sub>) were placed in methanol-D<sub>4</sub> with 0.1% formic acid to accelerate any potential exchange and were then assessed by <sup>1</sup>H NMR. Only Progesterone-D<sub>9</sub> was expected to show exchange.
- After 14 days at room temperature, only Progesterone-D<sub>9</sub> showed deuterium exchange under the harsh acidic conditions.
- No exchange was observed in Progesterone-D<sub>9</sub> after 10 days at room temperature in the absence of 0.1% formic acid.
- Exchange in solution would not be expected to occur under normal diluent or LC-MS/MS analysis conditions.



Progesterone-D<sub>9</sub> in the presence of 0.1% formic acid/methanol-D<sub>4</sub> over 2 weeks

## CONCLUSIONS

- Scrambling was observed on both tandem quad and QToF MS at select transitions for the deuterium-labeled internal standards studied. Ion source and diluent selection represent potential sources for scrambling/exchange.
- Scrambling can be mitigated by selection of an appropriate transition with the exception of Cortisol-D<sub>2</sub>.
- Awareness of potential scrambling is important for proper internal standard design and selection. Scrambling can be mitigated or eliminated by altering instrument conditions and transition selection. This approach is important in clinical method development to ensure accurate quantitation and reproducible results for critical decision-making in patient care.
- Deuterium-labeled internal standards are a viable option for LC-MS/MS analysis with selection of the appropriate transition. They also offer a more cost-effective alternative to carbon-13 or nitrogen-15-labeled analogs with benefits such as ready availability and lower cost per test.