

# Evaluation of LC-MS/MS Scrambling Ratios for Deuterium-Labeled Vitamin D Metabolites, Steroids and Other Compounds of Clinical Significance

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## Abstract

**Introduction and Objective:** A significant clinical challenge with LC-MS/MS is the potential for matrix effects that cause interferences or impact ionization efficiency. Stable isotope-labeled internal standards are frequently used to compensate for matrix effects and to increase the accuracy of quantitation. The use of a labeled internal standard that co-elutes with the drug being monitored can potentially 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 the collision cell at the selected transitions or in the ion source. In this study, we examined deuterium labeled 25-Hydroxyvitamin D, testosterone, and other compounds of clinical significance by LC-MS/MS at multiple transitions. We investigated reproducibility of the scrambling ratio and influences on scrambling of different LC-MS systems (tandem quadrupole vs. quadrupole time-of-flight), matrix selection, concentration, and deuterium placement in the internal standard.

## Methods and Procedures

LCMS System 1:

Instrument: Waters Alliance UPLC-Xevo G2 Q-ToF  
 Column: Waters Acquity UPLC, BEH C18, 1.7µm, 2.1 x 50mm

25-Hydroxyvitamin D Analysis Conditions:

UPLC Conditions: 0.4mL/min, gradient, 0.1:99.9 to 99.9:0.1 (0.1% formic acid in acetonitrile:0.1% formic acid in water)  
 MS Conditions: ESI+, Cone 25V, Capillary 2.5kV, CE 20

Testosterone Analysis Conditions:

UPLC Conditions: 0.4mL/min, isocratic, 30:70 (0.1% formic acid in acetonitrile:0.1% formic acid in water)  
 MS Conditions: ESI+, Cone 30V, Capillary 3.0kV, CE 18

LCMS System 2:

Instrument: Agilent 1100 HPLC-6410 triple quad  
 Column: Phenomenex Kinetex, C18, 3µm, 2.1 x 50mm

25-Hydroxyvitamin D Analysis Conditions:

HPLC Conditions: 0.4mL/min, isocratic, 80:20 (0.1% formic acid in methanol:0.1% formic acid in water)  
 MS Conditions: ESI+, Fragmentor 110V, Capillary 4.0kV, CE 5

Testosterone Analysis Conditions:

UPLC Conditions: 0.4mL/min, isocratic, 30:70 (0.1% formic acid in acetonitrile:0.1% formic acid in water)  
 MS Conditions: ESI+, Fragmentor 50V, Capillary 4.0kV, CE 10

Solution Standards Used:

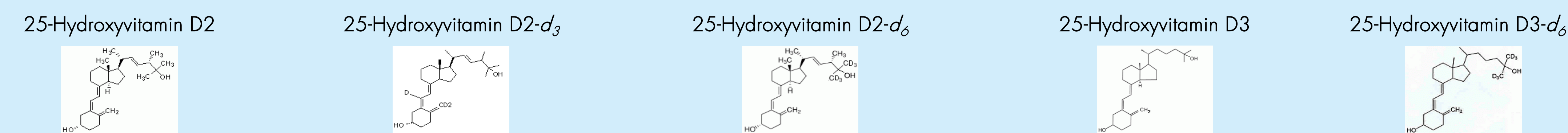
25-Hydroxyvitamin D3, Cat# H-083  
 25-Hydroxyvitamin D3-d<sub>6</sub>, Cat# H-074  
 25-Hydroxyvitamin D2, Cat# H-073  
 Testosterone, Cat# T-037  
 Testosterone-d<sub>3</sub>, Cat# T-046  
 Testosterone-<sup>13</sup>C<sub>3</sub>, Cat# T-037  
 Progesterone-d<sub>6</sub>, Cat# P-070  
 Pregabalin-d<sub>6</sub>, Cat# P-072

Serum Extraction:

200µL of sample in serum + 200µL of methanol, vortexed to mix. Added 1mL of heptane, vortexed for 30sec, centrifuged for 4min at 3000rpm. 900µL of top layer dried under nitrogen. Reconstituted in 100µL of ethanol

## Comparisons of 25-Hydroxyvitamin D2 and D3 Deuterium Scrambling

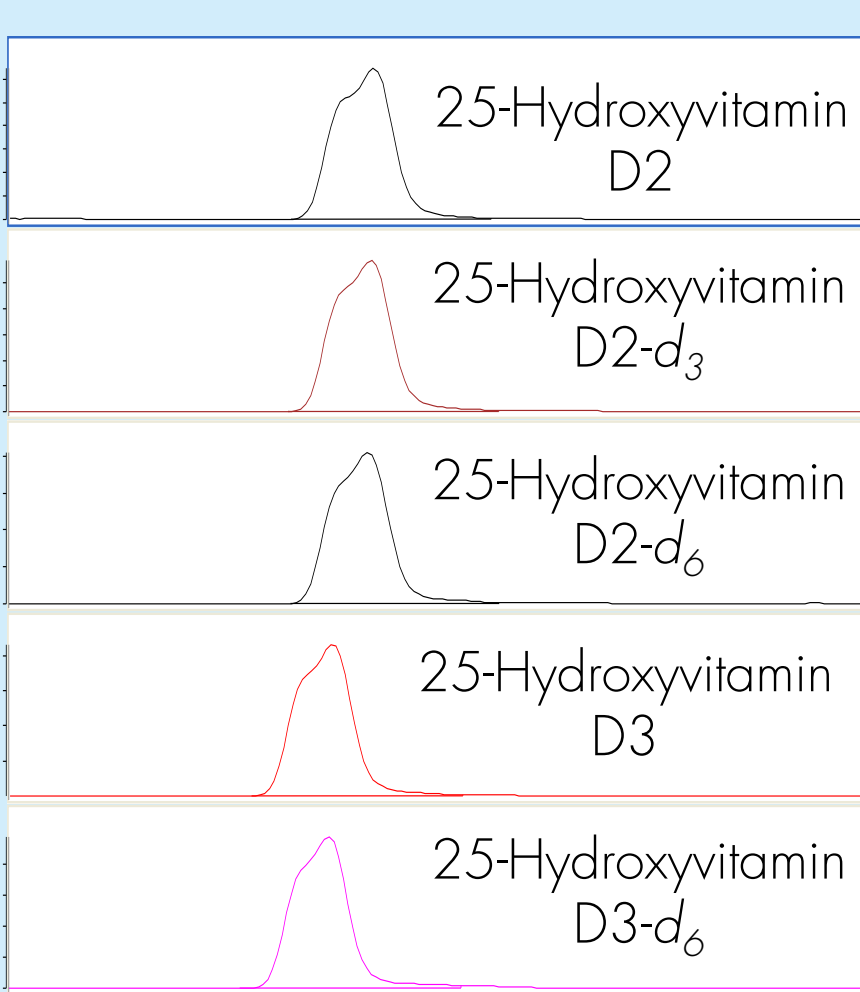
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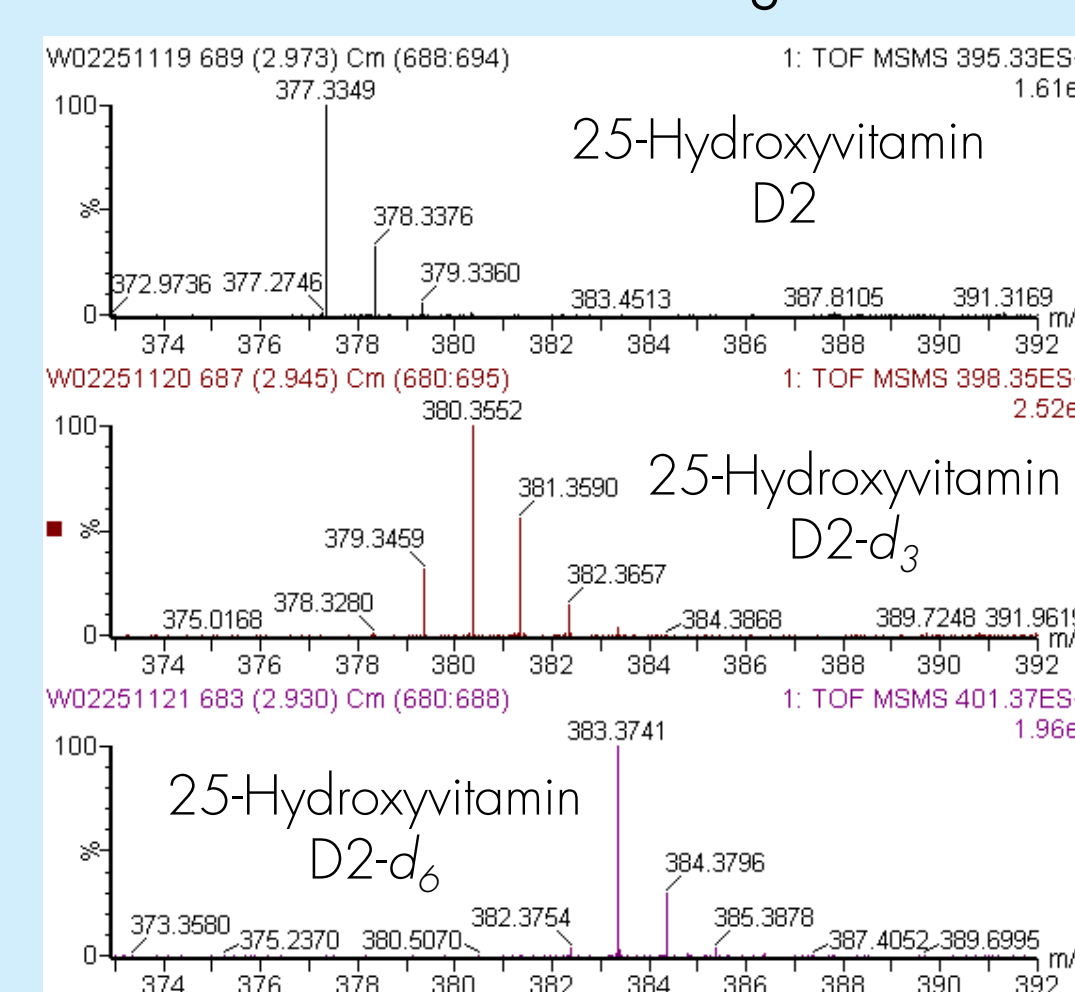
Labeled 25-Hydroxyvitamin D2 and D3 Scrambling in Serum

Compound	Label	System	Concentration µg/mL	Transition d <sub>n-1</sub>	Transition d <sub>n</sub>	Scrambling % d <sub>n-1</sub> / d <sub>n</sub>	
25-Hydroxyvitamin D2	d <sub>3</sub>	Xevo G2	2	398→379	398→380	28.6	
			0.2	398→379	398→380	35.4	
		6410	5	416→397	416→398	2.8	
				416→379	416→380	19.7	
				398→379	398→380	30.4	
				416→397	416→398	2.8	
	6410	50	416→379	416→380	20		
			398→379	398→380	30.5		
		d <sub>6</sub>	6410	5	419→400	419→401	2
					419→382	419→383	8.8
				401→382	401→383	5.9	
				419→400	419→401	2	
	419→382		419→383	9			
	401→382		401→383	5.4			
25-Hydroxyvitamin D3	d <sub>6</sub>	6410	2.5	407→388	407→389	4	
				407→370	407→371	18.8	
				389→370	389→371	9.2	

Vitamin D in Serum on 6410



Vitamin D in EtOH Scrambling on Xevo G2



Investigation of Method, Instrument, and Concentration Effects on Scrambling for Vitamin D

Compound	Method	Instrument	Concentration µg/mL	Transition d <sub>n-1</sub>	Transition d <sub>n</sub>	Scrambling % d <sub>n-1</sub> / d <sub>n</sub>
d <sub>3</sub> labeled 25-Hydroxyvitamin D2	Infusion	Q-ToF	10	398→379	398→380	29.7
			5			30.9
			10			27.1
	6410	LC	100	30.4		
			33	30.2		

Transitions Comparisons for Native and Labeled 25-Hydroxyvitamin D2 and D3 in EtOH on 6410

Parent → Water loss						
Compound	Label	Concentration µg/mL	Transition d <sub>n-1</sub>	Transition d <sub>n</sub>	Scrambling % d <sub>n-1</sub> / d <sub>n</sub>	
25-Hydroxyvitamin D2	d <sub>3</sub>	100	416→397	416→398	2.9	
	d <sub>6</sub>	100	419→400	419→401	2	
	native	50	413→394	413→395	0.5	
25-Hydroxyvitamin D3	d <sub>6</sub>	50	407→388	407→389	4	
	native	100	401→382	401→383	0.5	

Parent → 2 Water losses						
Compound	Label	Concentration µg/mL	Transition d <sub>n-1</sub>	Transition d <sub>n</sub>	Scrambling % d <sub>n-1</sub> / d <sub>n</sub>	
25-Hydroxyvitamin D2	d <sub>3</sub>	100	416→379	416→380	19.5	
	d <sub>6</sub>	100	419→382	419→383	8.9	
	native	50	413→376	413→377	0.5	
25-Hydroxyvitamin D3	d <sub>6</sub>	50	407→370	407→371	18.9	
	native	100	401→364	401→365	0.3	

Water Loss → 2 Water losses						
Compound	Label	Concentration µg/mL	Transition d <sub>n-1</sub>	Transition d <sub>n</sub>	Scrambling % d <sub>n-1</sub> / d <sub>n</sub>	
25-Hydroxyvitamin D2	d <sub>3</sub>	100	398→379	398→380	30.4	
	d <sub>6</sub>	100	401→382	401→383	5.4	
	native	50	398→376	398→377	0.4	
25-Hydroxyvitamin D3	d <sub>6</sub>	50	389→370	389→371	11.2	
	native	100	383→364	383→365	0.3	

Notes: 25-Hydroxy D2-D6 water loss→2 water loss has same transition as 25-Hydroxyvitamin D3 parent→water loss. Can be problem if compounds are not well resolved chromatographically

Selection of Transitions Greatly Impacts Observed Scrambling

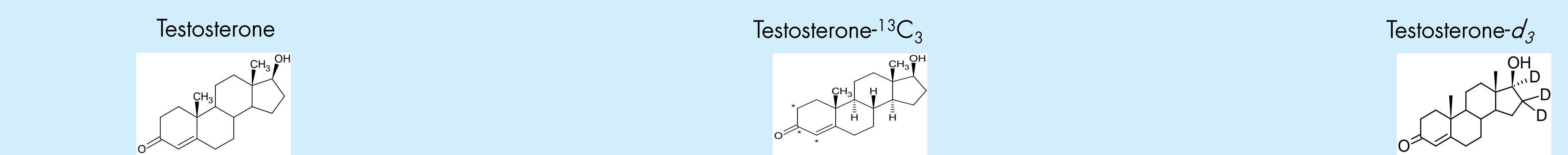
5µg/mL Infusion at 20µL/min of d<sub>3</sub> labeled 25-Hydroxyvitamin D2 on Xevo G2

Transition d <sub>n-1</sub>	Transition d <sub>n</sub>	Scrambling % d <sub>n-1</sub> / d <sub>n</sub>
416→397	416→398	2.2
416→379	416→380	16.9
398→379	398→380	30.9

Note: Under optimized UPLC-Q-ToF conditions only water loss MS ions were detected. MS ion ratios changed for 25-Hydroxyvitamin D when combined with mobile phase. Could detect ions without water loss when infusing.

## Investigation of Testosterone Scrambling

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Testosterone Chromatograms on 6410

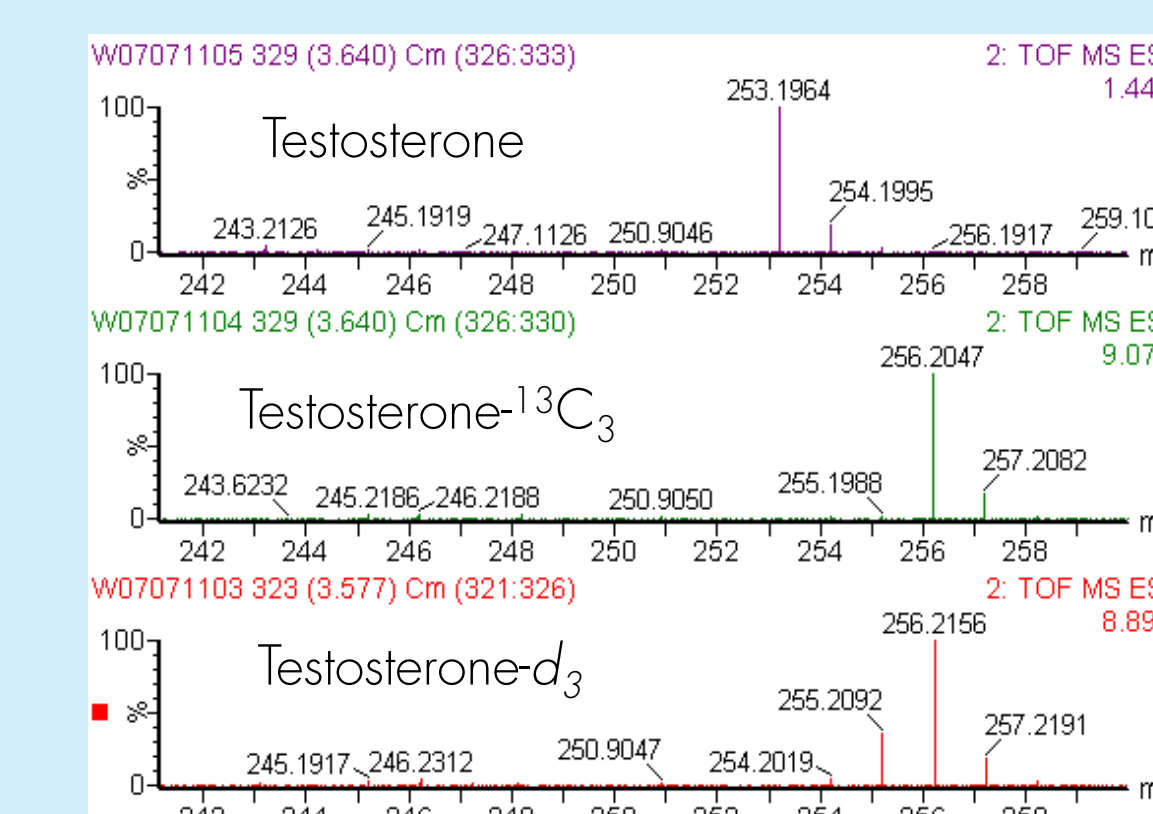


Testosterone Scrambling Comparison

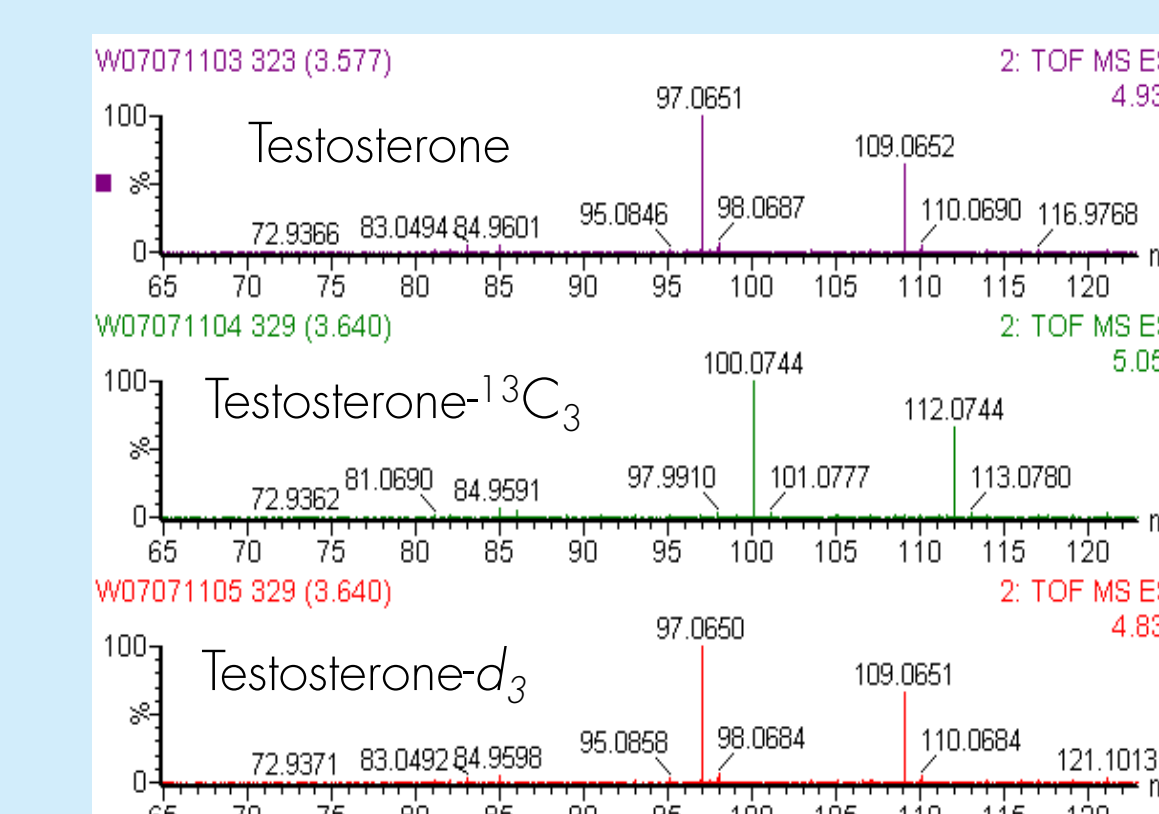
Label	Method	Instrument	Concentration µg/mL	Transitions D <sub>n-1</sub> or <sup>13</sup> C <sub>n-1</sub>	Transitions D <sub>n</sub> or <sup>13</sup> C <sub>n</sub>	*Scrambling % D <sub>n-1</sub> / D <sub>n</sub>
d <sub>3</sub>	Infusion	Q-ToF	10	292→255	292→256	31.9
			100			36.5
			10			35.7
	6410	LC	100	37.7		
			10	36.3		
			100	0.1		
native		100	289→252	289→253	0.0	

Major transitions are:  
 Native: 289→97 & 289→109  
 Testosterone-d<sub>3</sub>: 292→97 & 292→109  
 Testosterone-<sup>13</sup>C<sub>3</sub>: 292→100 & 292→112  
 No scrambling at major transitions

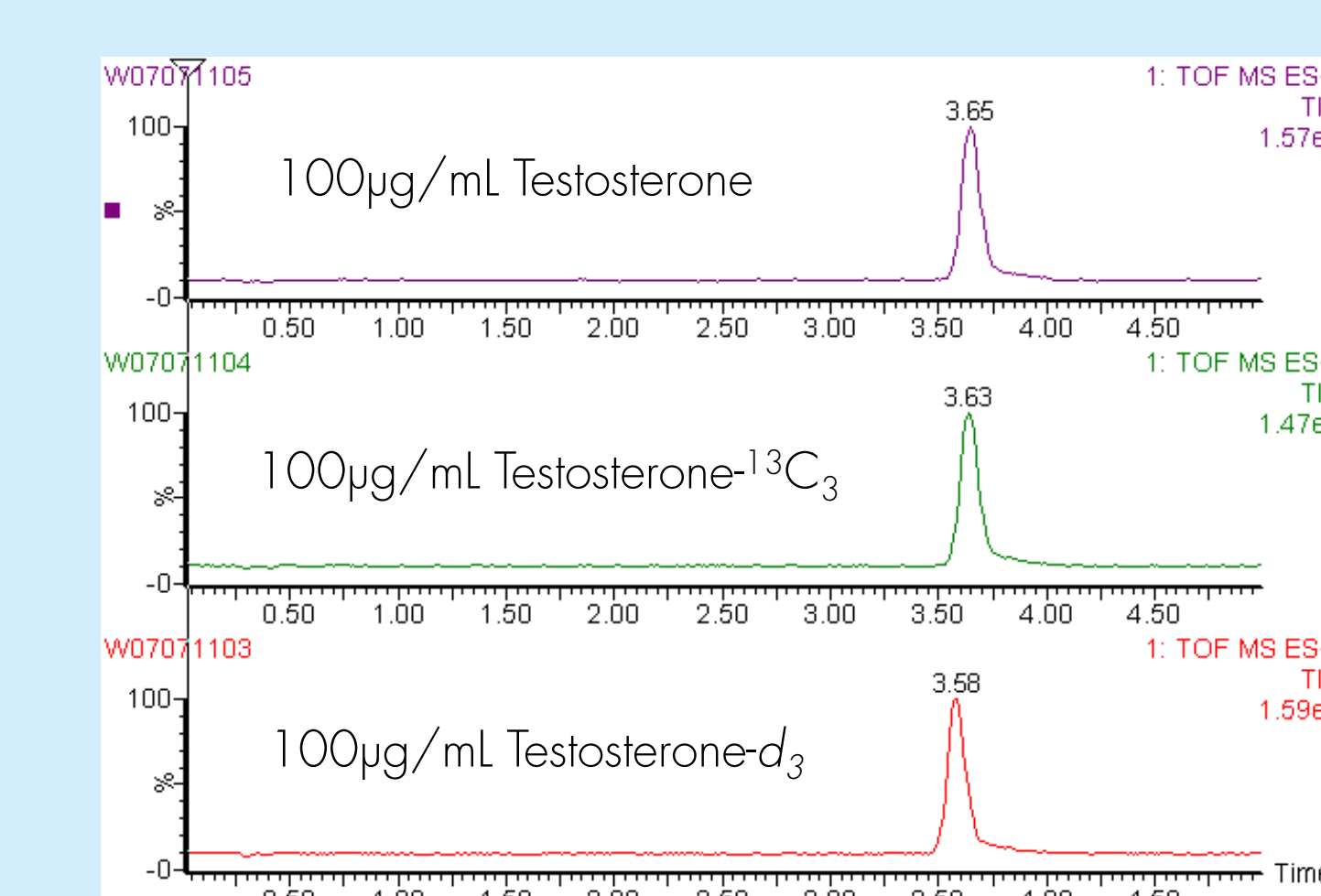
Testosterone Scrambling at m/z 253



Testosterone Scrambling at 97 and 109



Testosterone Chromatograms on Xevo G2



Testosterone d<sub>n-2</sub> / d<sub>n</sub> Scrambling

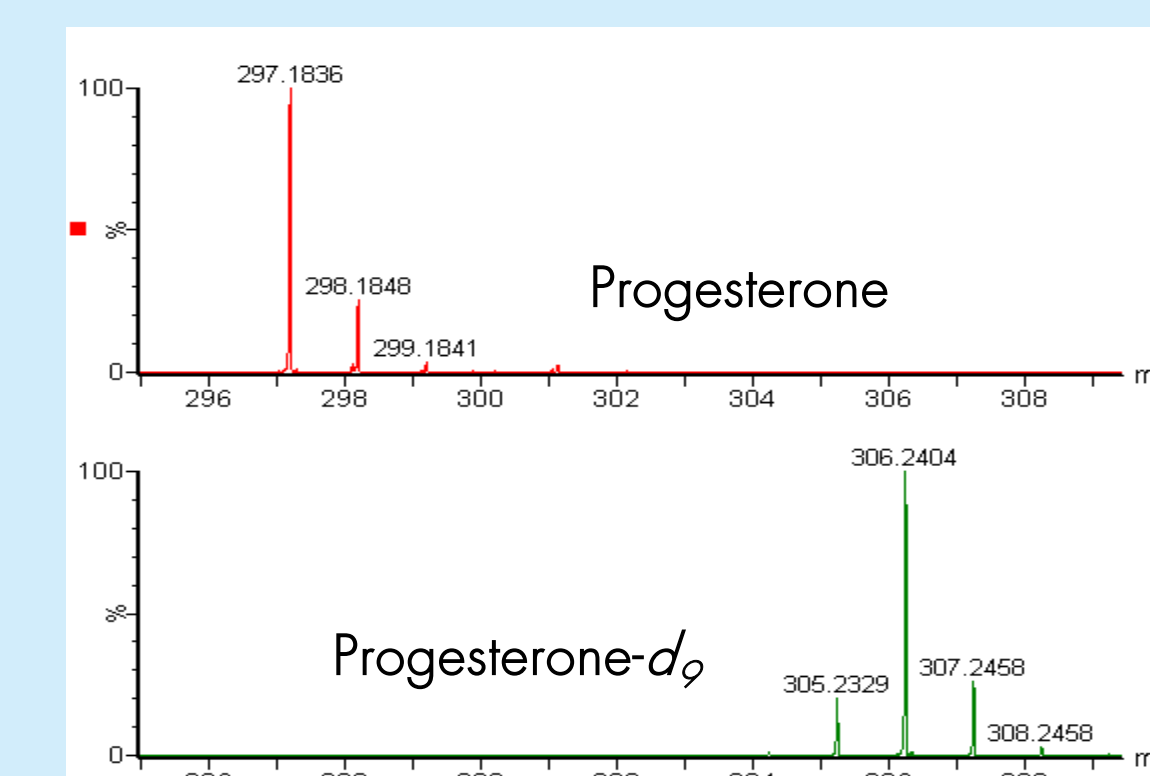
Label	Method	Instrument	Concentration µg/mL	Transition d <sub>n-2</sub>	Transition d <sub>n</sub>	Scrambling % d <sub>n-2</sub> / d <sub>n</sub>
d <sub>3</sub>	Infusion	Q-ToF	10	292→254	292→256	2.6
d <sub>3</sub>	LC	Q-ToF	100	292→254	292→256	3.6
d <sub>3</sub>	LC	Q-ToF	10	292→254	292→256	<LOD

## Scrambling for other clinical compounds

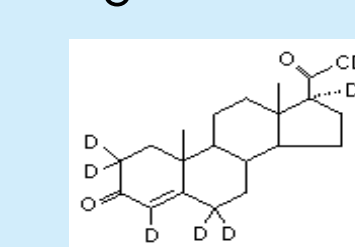
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Xevo G2 Scrambling Infusion Experiments

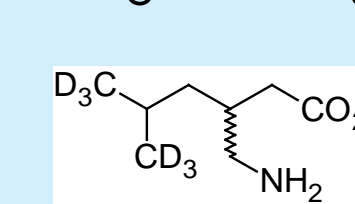
Compound	Label	Transition d <sub>n-1</sub>	Transition d <sub>n</sub>	Scrambling % d <sub>n-1</sub> / d <sub>n</sub>	Transition d <sub>n-1</sub>
Progesterone	d <sub>6</sub>	324→305	324→306	20	19
		324→287	324→288	77	19
		324→112	324→113	0	19
		324→99	324→100	0	19
Pregabalin	d <sub>6</sub>	166→147	166→148	0	25
		166→129	166→130	0	25
		166→102	166→103	12	25
		166→88	166→89	40	25



Progesterone-d<sub>6</sub>



Pregabalin-d<sub>6</sub>



## CONCLUSIONS

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- Scrambling was observed on both the Agilent 6410 triple quadrupole and the Waters Xevo G2 Q-ToF, and in some cases was very pronounced.
- For a specific transition, scrambling ratios were consistent between solvent and serum. No matrix effects on scrambling.
- Direct infusion can provide rapid and accurate determination of scrambling ratios. Infusion and chromatographic injection results were consistent.
- It may be advisable to investigate at higher concentrations than normally analyzed to ensure that instrument sensitivity does not impact accuracy of scrambling determination.
- Awareness of potential scrambling is important for proper internal standard selection. Scrambling may be mitigated or eliminated by altering instrument conditions and transition selection.
- Deuterium-labeled internal standards are a viable option for LC-MS/MS analysis with selection of the appropriate transition. Deuterated standards can be more cost effective than <sup>13</sup>C labeled internal standards, more widely available and with lower cost per test. <sup>13</sup>C labeled internal standards are most effective when deuterium scrambling issues can not be resolved.