

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, 1.7 μ m, 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, 3 μ m, 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

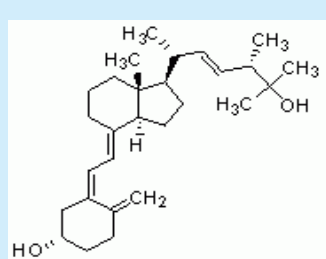
Solution Standards Used:

25-Hydroxyvitamin D3, Cat# H-083
25-Hydroxyvitamin D3-d₆, Cat# H-074
25-Hydroxyvitamin D2, Cat# H-073
Testosterone, Cat# T-037
Testosterone-d₃, Cat# T-046
Testosterone-¹³C₃, Cat# T-037
Progesterone-d₆, Cat# P-070
Pregabalin-d₆, Cat# P-072

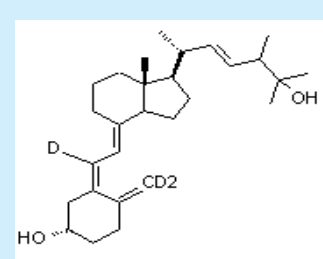
Serum Extraction:
200 μ L of sample in serum + 200 μ L of methanol, vortexed to mix.
Added 1mL of heptane, vortexed for 30sec,
Centrifuged for 4min at 3000rpm
900 μ L of top layer dried under nitrogen
Reconstituted in 100 μ L of ethanol

Comparisons of 25-Hydroxyvitamin D2 and D3 Deuterium Scrambling

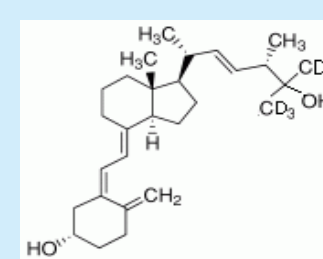
25-Hydroxyvitamin D2



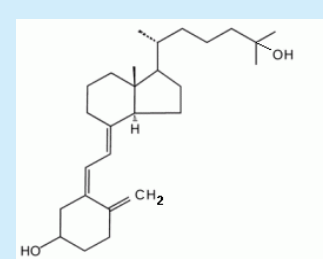
25-Hydroxyvitamin D2-d₃



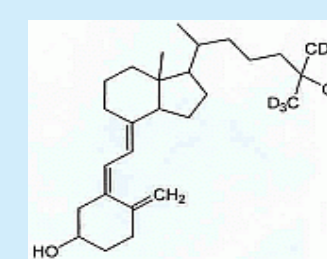
25-Hydroxyvitamin D2-d₆



25-Hydroxyvitamin D3



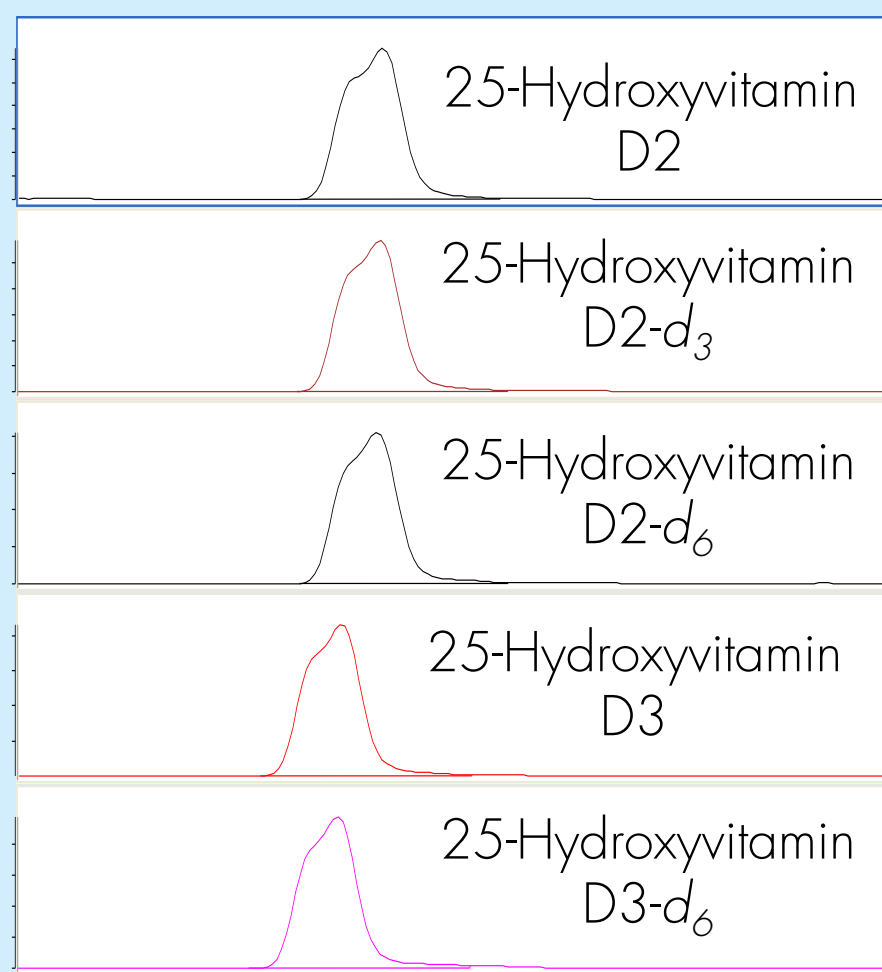
25-Hydroxyvitamin D3-d₆



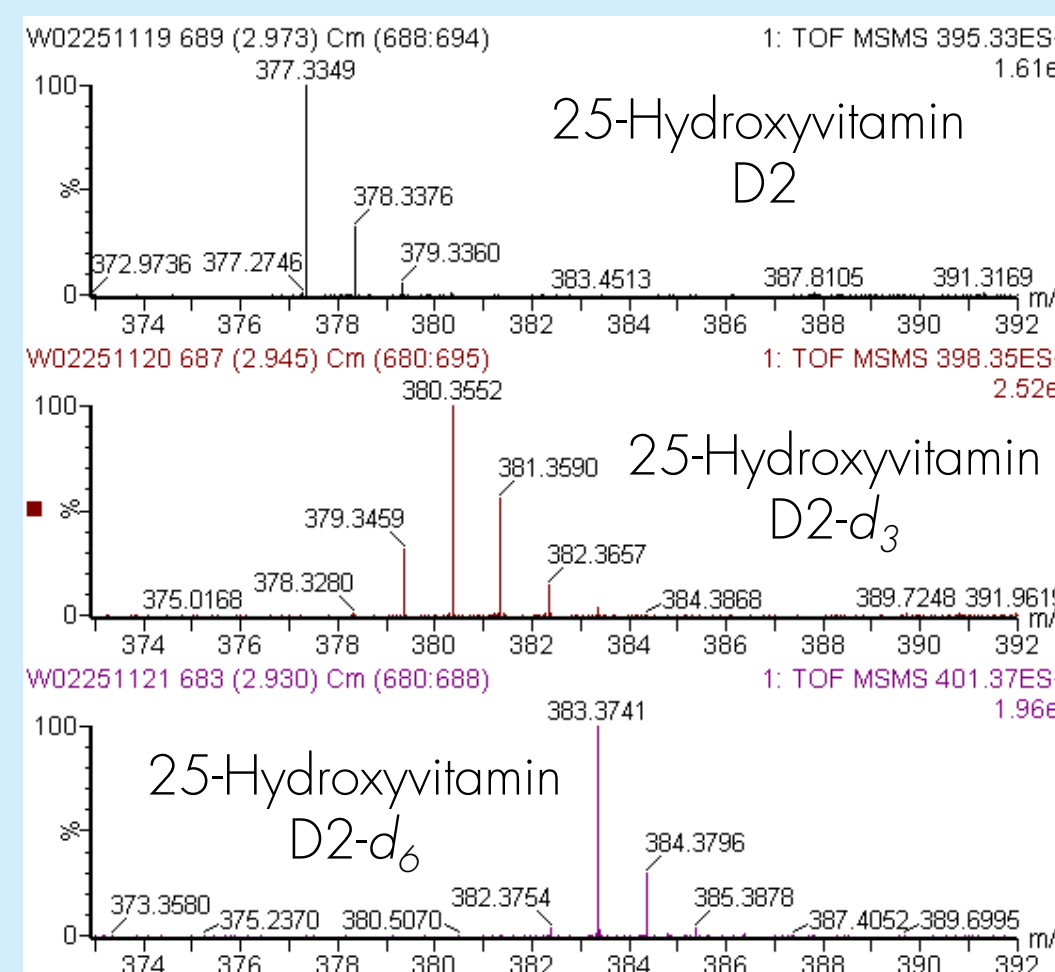
Labeled 25-Hydroxyvitamin D2 and D3 Scrambling in Serum

Compound	Label	System	Concentration μ g/mL	Transition d_{n-1}	Transition d_n	Scrambling % d_{n-1} / d_n
25-Hydroxyvitamin D2	d_3	Xevo G2	2	398 \rightarrow 379	398 \rightarrow 380	28.6
			0.2	398 \rightarrow 379	398 \rightarrow 380	35.4
			5	416 \rightarrow 397	416 \rightarrow 398	2.8
				416 \rightarrow 379	416 \rightarrow 380	19.7
				398 \rightarrow 379	398 \rightarrow 380	30.4
				416 \rightarrow 397	416 \rightarrow 398	2.8
	d_6	6410	50	416 \rightarrow 379	416 \rightarrow 380	20
				398 \rightarrow 379	398 \rightarrow 380	30.5
				419 \rightarrow 400	419 \rightarrow 401	2
				419 \rightarrow 382	419 \rightarrow 383	8.8
			5	401 \rightarrow 382	401 \rightarrow 383	5.9
				419 \rightarrow 400	419 \rightarrow 401	2
25-Hydroxyvitamin D3	d_6	6410	50	419 \rightarrow 382	419 \rightarrow 383	9
				401 \rightarrow 382	401 \rightarrow 383	5.4
				407 \rightarrow 388	407 \rightarrow 389	4
				407 \rightarrow 370	407 \rightarrow 371	18.8
			2.5	389 \rightarrow 370	389 \rightarrow 371	9.2

Vitamin D in Serum on 6410



Vitamin D in EtOH Scrambling on Xevo G2



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

Parent \rightarrow Water loss					
Compound	Label	Concentration μ g/mL	Transition d_{n-1}	Transition d_n	Scrambling % d_{n-1} / d_n
25-Hydroxyvitamin D2	d_3	100	416 \rightarrow 397	416 \rightarrow 398	2.9
	d_6	100	419 \rightarrow 400	419 \rightarrow 401	2
	native	50	413 \rightarrow 394	413 \rightarrow 395	0.5
25-Hydroxyvitamin D3	d_6	50	407 \rightarrow 388	407 \rightarrow 389	4
	native	100	401 \rightarrow 382	401 \rightarrow 383	0.5

Parent \rightarrow 2 Water losses

Compound	Label	Concentration μ g/mL	Transition d_{n-1}	Transition d_n	Scrambling % d_{n-1} / d_n
25-Hydroxyvitamin D2	d_3	100	416 \rightarrow 379	416 \rightarrow 380	19.5
	d_6	100	419 \rightarrow 382	419 \rightarrow 383	8.9
	native	50	413 \rightarrow 376	413 \rightarrow 377	0.5
25-Hydroxyvitamin D3	d_6	50	407 \rightarrow 370	407 \rightarrow 371	18.9
	native	100	401 \rightarrow 364	401 \rightarrow 365	0.3

Water Loss \rightarrow 2 Water losses

Compound	Label	Concentration μ g/mL	Transition d_{n-1}	Transition d_n	Scrambling % d_{n-1} / d_n
25-Hydroxyvitamin D2	d_3	100	398 \rightarrow 379	398 \rightarrow 380	30.4
	d_6	100	401 \rightarrow 382	401 \rightarrow 383	5.4
	native	50	398 \rightarrow 376	398 \rightarrow 377	0.4
25-Hydroxyvitamin D3	d_6	50	389 \rightarrow 370	389 \rightarrow 371	11.2
	native	100	383 \rightarrow 364	383 \rightarrow 365	0.3

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

Selection of Transitions Greatly Impacts Observed Scrambling

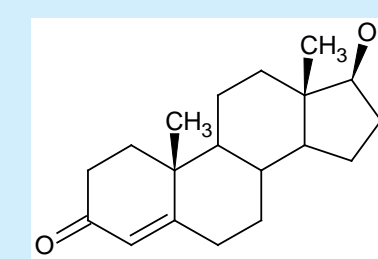
5 μ g/mL Infusion at 20 μ L/min of d_3 labeled 25-Hydroxyvitamin D2 on Xevo G2

Transition d_{n-1}	Transition d_n	Scrambling % d_{n-1} / d_n
416 \rightarrow 397	416 \rightarrow 398	2.2
416 \rightarrow 379	416 \rightarrow 380	16.9
398 \rightarrow 379	398 \rightarrow 380	30.9

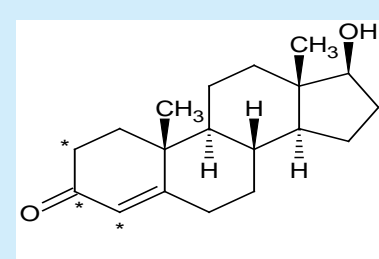
Note: Under optimized UPLC-Q-ToF conditions only water loss MS ions were detected. MS ion ratios changed for 25-Hydroxyvitamin D when combined with mobile phase. Could detect ions without water loss when infusing.

Investigation of Testosterone Scrambling

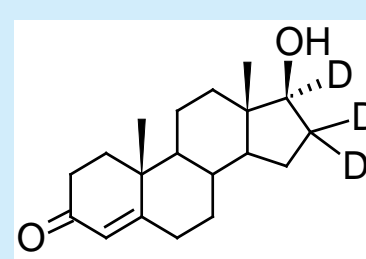
Testosterone



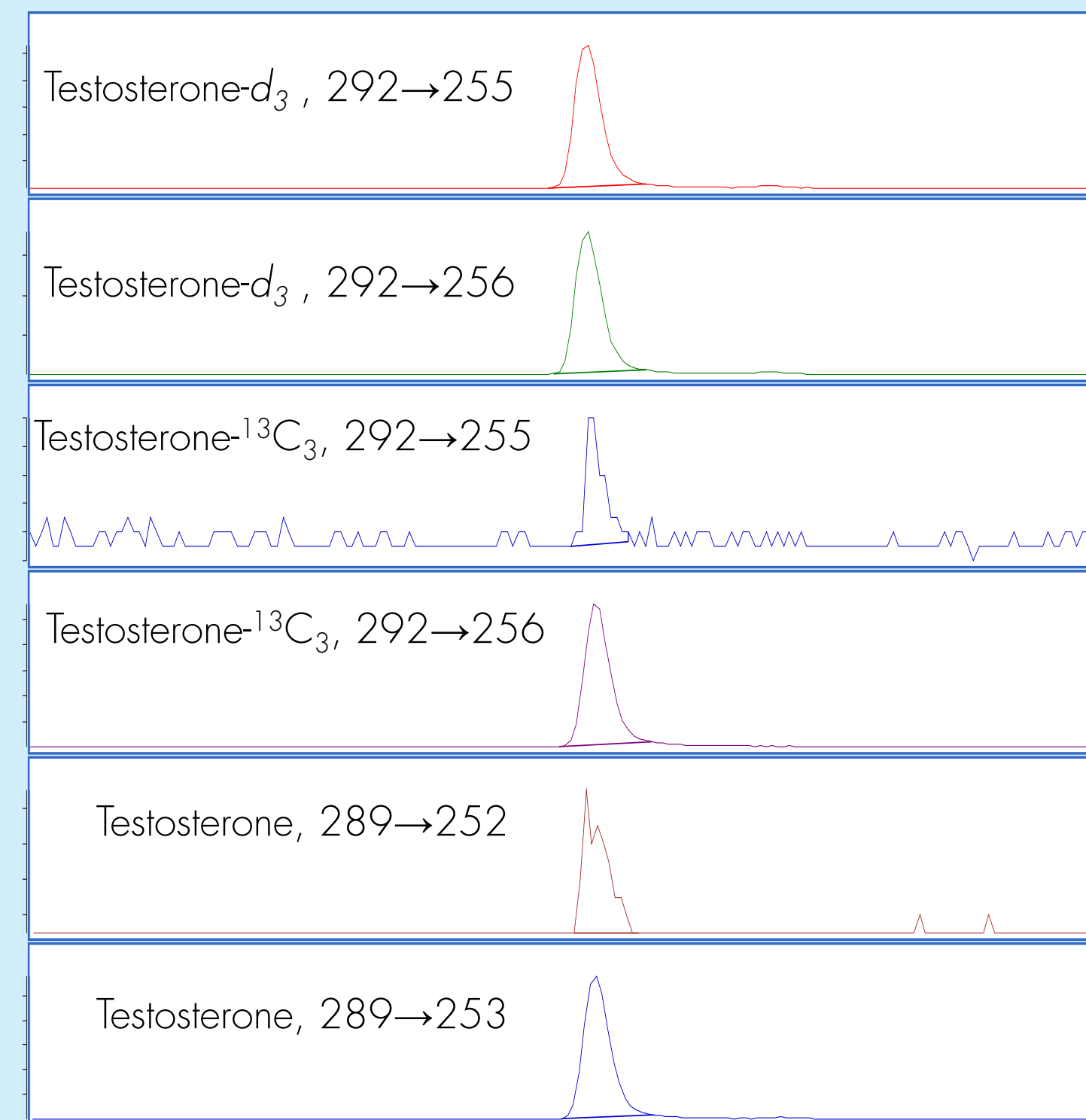
Testosterone-¹³C₃



Testosterone-d₃



Testosterone Chromatograms on 6410

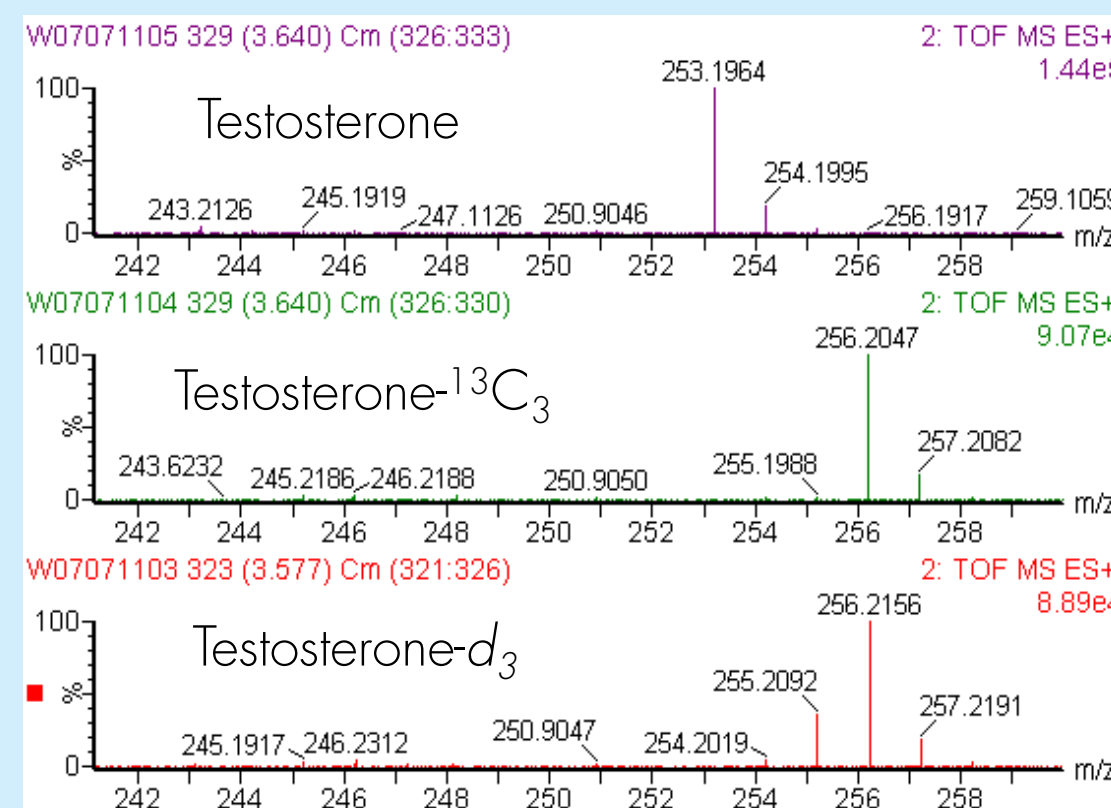


Testosterone Scrambling Comparison

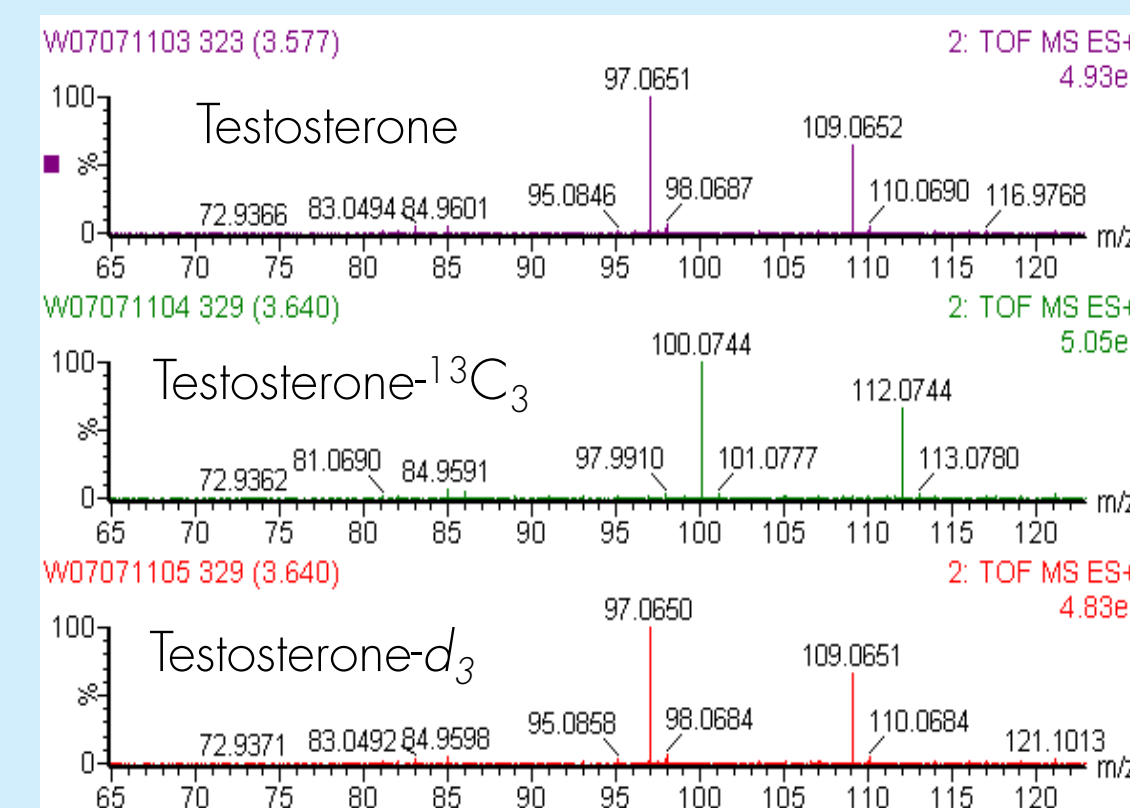
Label	Method	Instrument	Concentration μ g/mL	Transitions D_{n-1} or ¹³ C _{n-1}	Transitions D_n or ¹³ C _n	*Scrambling % D_{n-1} / D_n
d_3	Infusion	Q-ToF	10	292 \rightarrow 255	292 \rightarrow 256	31.9
			100			36.5
			10			35.7
	LC	6410	100			37.7
			10			36.3
			100			0.1
native			100	289 \rightarrow 252	289 \rightarrow 253	0.0

Major transitions are:
Native: 289 \rightarrow 97 & 289 \rightarrow 109
Testosterone-d₃: 292 \rightarrow 97 & 292 \rightarrow 109
Testosterone-¹³C₃: 292 \rightarrow 100 & 292 \rightarrow 112
No scrambling at major transitions

Testosterone Scrambling at m/z 253



Testosterone Scrambling at 97 and 109



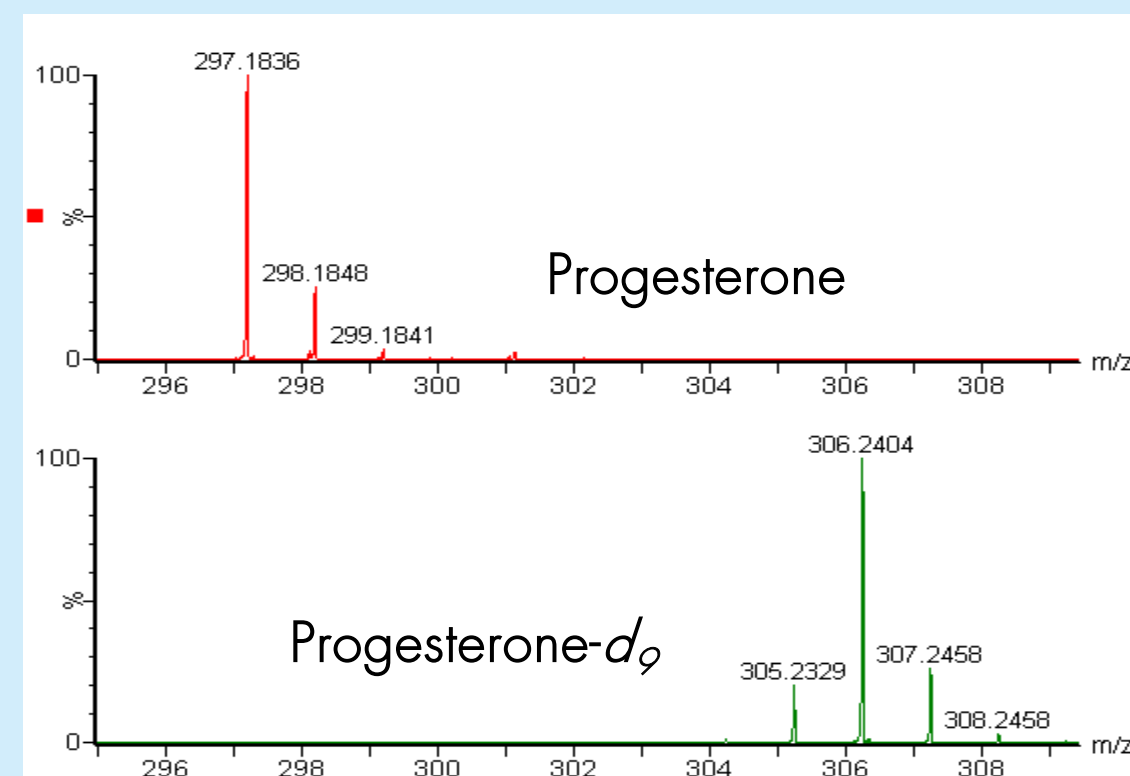
Testosterone d_{n-2} / d_n Scrambling

Label	Method	Instrument	Concentration μ g/mL	Transition d_{n-2}	Transition d_n	Scrambling % d_{n-2} / d_n
d_3	Infusion	Q-ToF	10	292 \rightarrow 254	292 \rightarrow 256	2.6
d_3	LC	Q-ToF	100	292 \rightarrow 254	292 \rightarrow 256	3.6
d_3	LC	Q-ToF	10	292 \rightarrow 254	292 \rightarrow 256	<LOD

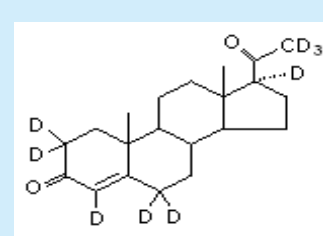
Scrambling for other clinical compounds

Xevo G2 Scrambling Infusion Experiments

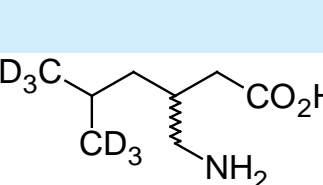
Compound	Label	Transition d_{n-1}	Transition d_n	Scrambling % d_{n-1} / d_n	Transition d_{n-1}
Progesterone	d_6	324 \rightarrow 305	324 \rightarrow 306	20	19
		324 \rightarrow 287	324 \rightarrow 288	77	19
		324 \rightarrow 112	324 \rightarrow 113	0	19
		324 \rightarrow 99	324 \rightarrow 100	0	19
Pregabalin	d_6	166 \rightarrow 147	166 \rightarrow 148	0	25
		166 \rightarrow 129	166 \rightarrow 130	0	25
		166 \rightarrow 102	166 \rightarrow 103	12	25
		166 \rightarrow 88	166 \rightarrow 89	40	25



Progesterone-d₆



Pregabalin-d₆



CONCLUSIONS

- 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 ¹³C labeled internal standards, more widely available and with lower cost per test. ¹³C labeled internal standards are most effective when deuterium scrambling issues can not be resolved.