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 1

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 2

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 99.9:01 (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_6, Cat# H-074
25-Hydroxyvitamin D2, Cat# H-073
Testosterone, Cat# T-037
Testosterone-d_3, Cat# T-046
Testosterone-$^{13}C_3$, Cat# T-037
Progesterone-d_9, Cat# P-070
Pregabalin-d_6, Cat# P-072

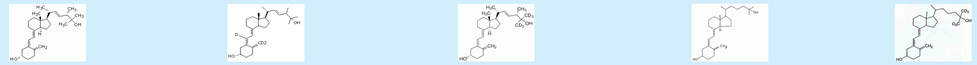
Serum Extraction:
200µL of sample in serum + 200µL of methanol, vortexed to mix.
Added 1mL of heptane, vortexed for 30sec,
Centrifuged for 4min at 3000rpm
900µL of top layer dried under nitrogen
Reconstituted in 100µL of ethanol

Cerilliant® Analytical Reference Standards
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Comparisons of 25-Hydroxyvitamin D2 and D3 Deuterium Scrambling 3

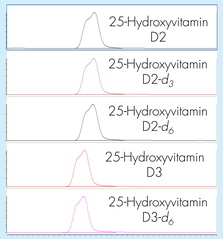
25-Hydroxyvitamin D2 | 25-Hydroxyvitamin D2-d_3 | 25-Hydroxyvitamin D2-d_6 | 25-Hydroxyvitamin D3 | 25-Hydroxyvitamin D3-d_6



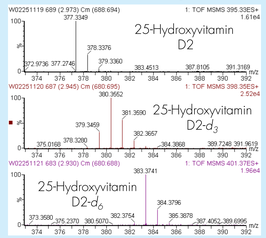
Labeled 25-Hydroxyvitamin D2 and D3 Scrambling in Serum

Compound	Label	System	Concentration µg/mL	Transition d_{n-1}	Transition d_n	Scrambling % d_{n-1} / d_n
25-Hydroxyvitamin D2	d_3	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
			50	416→397	416→398	2.8
				416→379	416→380	20
				398→379	398→380	30.5
	d_6	6410	5	419→400	419→401	2
				419→382	419→383	8.8
				401→382	401→383	5.9
			50	419→400	419→401	2
				419→382	419→383	9
				401→382	401→383	5.4
25-Hydroxyvitamin D3	d_6	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



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_{n-1}	Transition d_n	Scrambling % d_{n-1} / d_n
25-Hydroxyvitamin D2	d_3	100	416→397	416→398	2.9
	d_6	100	419→400	419→401	2
	native	50	413→394	413→395	0.5
25-Hydroxyvitamin D3	d_6	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_{n-1}	Transition d_n	Scrambling % d_{n-1} / d_n
25-Hydroxyvitamin D2	d_3	100	416→379	416→380	19.5
	d_6	100	419→382	419→383	8.9
	native	50	413→376	413→377	0.5
25-Hydroxyvitamin D3	d_6	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_{n-1}	Transition d_n	Scrambling % d_{n-1} / d_n
25-Hydroxyvitamin D2	d_3	100	398→379	398→380	30.4
	d_6	100	401→382	401→383	5.4
	native	50	398→376	398→377	0.4
25-Hydroxyvitamin D3	d_6	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

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

Compound	Method	Instrument	Concentration µg/mL	Transition d_{n-1}	Transition d_n	Scrambling % d_{n-1} / d_n
d_3 labeled 25-Hydroxyvitamin D2	Infusion	Q-Tof	10	398→379	398→380	29.7
			5			30.9
	LC		10			27.1
		6410	100			30.4
			33			30.2

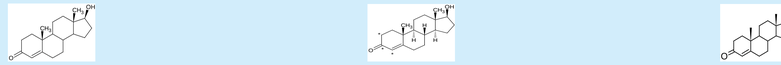
5µg/mL Infusion at 20µL/min of d_3 labeled 25-Hydroxyvitamin D2 on Xevo G2

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

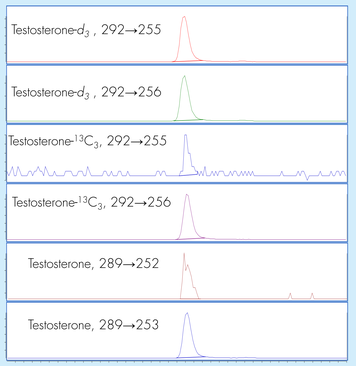
Note: Under optimized UPLC-Q-Tof conditions only water loss MS ions were detected. MS ion ratios changed for 25-Hydroxyvitamin D when combined with mobile phase. Could detect ions without water loss when infusing.

Investigation of Testosterone Scrambling 4

Testosterone | Testosterone-$^{13}C_3$ | Testosterone-d_3



Testosterone Chromatograms on 6410



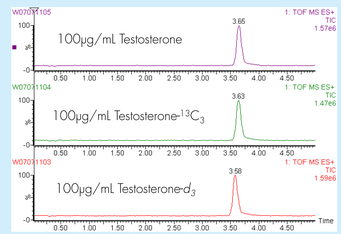
Testosterone Scrambling Comparison

Label	Method	Instrument	Concentration µg/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→255	292→256	31.9
	LC		100			36.5
		6410	10			35.7
			100			37.7
			10			36.3
$^{13}C_3$			100			0.1
native			100	289→252	289→253	0.0

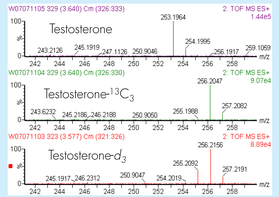
* or Scrambling % $^{13}C_{n-1}$ / $^{13}C_n$

Major transitions are:
Native: 289→97 & 289→109
Testosterone-d_3: 292→97 & 292→109
Testosterone-$^{13}C_3$: 292→100 & 292→112
No scrambling at major transitions

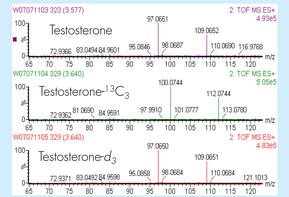
Testosterone Chromatograms on Xevo G2



Testosterone Scrambling at m/z 253



Testosterone Scrambling at 97 and 109



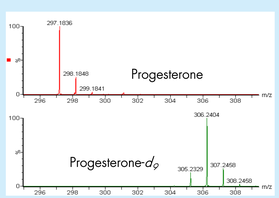
Testosterone d_{n-2} / d_n Scrambling

Label	Method	Instrument	Concentration µg/mL	Transition d_{n-2}	Transition d_n	Scrambling % d_{n-2} / d_n
d_2	Infusion	Q-Tof	10	292→254	292→256	2.6
d_2	LC	Q-Tof	100	292→254	292→256	3.6
d_3	LC	Q-Tof	10	292→254	292→256	<LOD

Scrambling for other clinical compounds 5

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_9	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_6	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_9



Pregabalin-d_6



CONCLUSIONS 6

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