# 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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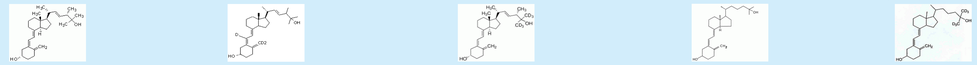
## Comparisons of 25-Hydroxyvitamin D2 and D3 Deuterium Scrambling 3

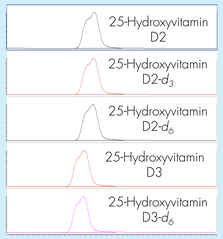
25-Hydroxyvitamin D2 | 25-Hydroxyvitamin D2-$d_3$ | 25-Hydroxyvitamin D2-$d_6$ | 25-Hydroxyvitamin D3 | 25-Hydroxyvitamin D3-$d_6$



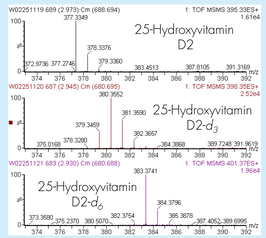
Labeled 25-Hydroxyvitamin D2 and D3 Scrambling in Serum

| 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 |

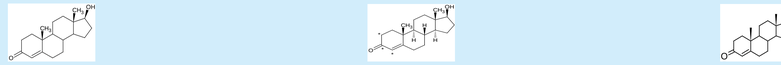
5µg/mL Infusion at 20µL/min of $d_3$ labeled 25-Hydroxyvitamin D2 on Xevo G2

| 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 |

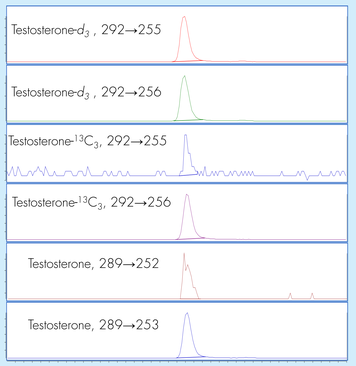
Note: Under optimized UPLC-Q-Tof conditions only water loss MS ions were detected. MS ion ratios changed for 25-Hydroxyvitamin D when combined with mobile phase. Could detect ions without water loss when infusing.

## Investigation of Testosterone Scrambling 4

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



Testosterone Chromatograms on 6410



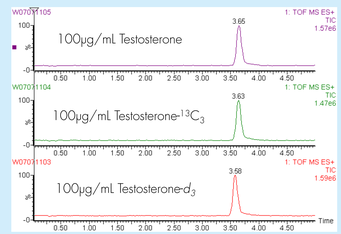

Testosterone Scrambling Comparison

| 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 |

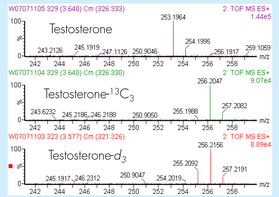
* or Scrambling % $^{13}C_{n-1}$ / $^{13}C_n$

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

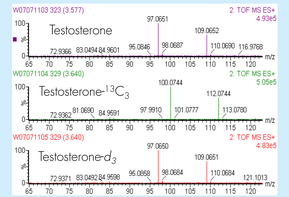
Testosterone Chromatograms on Xevo G2




Testosterone Scrambling at m/z 253




Testosterone Scrambling at 97 and 109



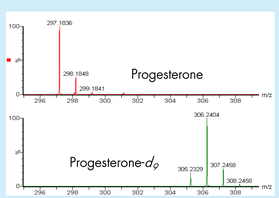

Testosterone $d_{n-2}$ / $d_n$ Scrambling

| 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.