

Selection of Internal Standards for LC-MS/MS Applications

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Selection Criteria for Internal Standards

Internal standards (IS) are utilized across a wide range of mass spectrometry applications including therapeutic drug monitoring, newborn screening, endocrinology, and pain management testing. They are especially useful in improving the accuracy of quantitation in complex matrices. Internal standards work by normalizing for differences in extraction, injection, chromatography, ionization and detection between samples.

Design Specifications/Requirements

Intended use	What is the mode of ionization and MS/MS platform?
Analyte	<ul style="list-style-type: none"> Ionization response and fragmentation pattern of IS similar to the analyte Adequate mass differentiation: IS MRM transitions should not interfere with analyte MRMs
Chromatography	<ul style="list-style-type: none"> Identical or close to analyte of interest for stable labeled analogs Chromatographically resolved from analyte for isobaric structural analogs
Purity	<ul style="list-style-type: none"> Impurities do not cause interferences with analyte or other analytes in the test panel (ie. isobaric, co-eluting impurities) Isotopic purity: ratio of M_0/M_n Isotopic distribution : $M_0...M_n, M_{n+1}, M_{n+2}$ Isotopic distribution should be adjusted for natural abundance of isotopes (important for analytes with Cl, Br, S) Isotopic distribution might vary from lot to lot. Caution is required when multiple lots of internal standard are used in the same analysis
Dynamic Range & Sensitivity	<ul style="list-style-type: none"> Methods with wide dynamic range and/or high sensitivity (lower quantitation levels) require internal standards that are high purity, co-elute with the analyte, and exhibit minimal or no scrambling or cross talk (e.g. steroids)

Types of Internal Standards

There are two main types of internal standards. Structural Analogs, which are similar in structure to an analyte, and Stable Label Analogs, which are typically Deuterium (D), ^{13}C or ^{15}N versions of an analyte.

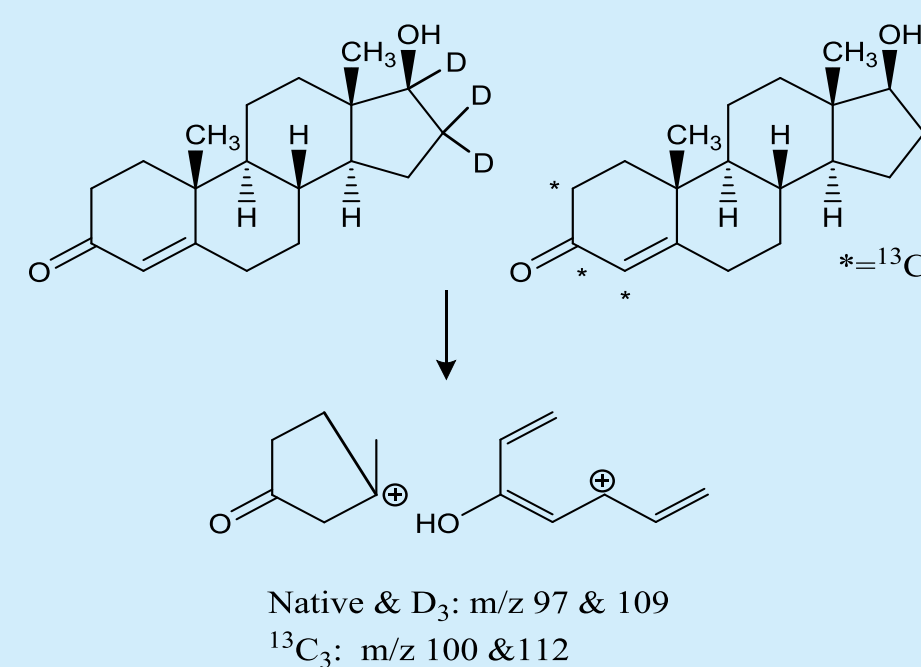
Structural Analogs

Isomers and structurally related compounds may be used as internal standards in MS applications:

- Ability to correct for matrix factors may be limited due to inherent differences from the analyte of interest with regard to ionization, chromatography and stability
- Used when suitable stable labeled analogs are not available
- Examples include Cyclosporin D and Ascomycin for immunosuppressant monitoring

Stable Labeled Analogs: D, ^{13}C , ^{15}N ...

- Choice of labeled internal standards depends on availability, isotope effects/scrambling, method LOQ, and sensitivity requirements of end use
- Availability and cost of stable labeled internal standards are limited primarily by reagent availability for chemical or biochemical synthesis, and synthesis complexity
- Examples include:



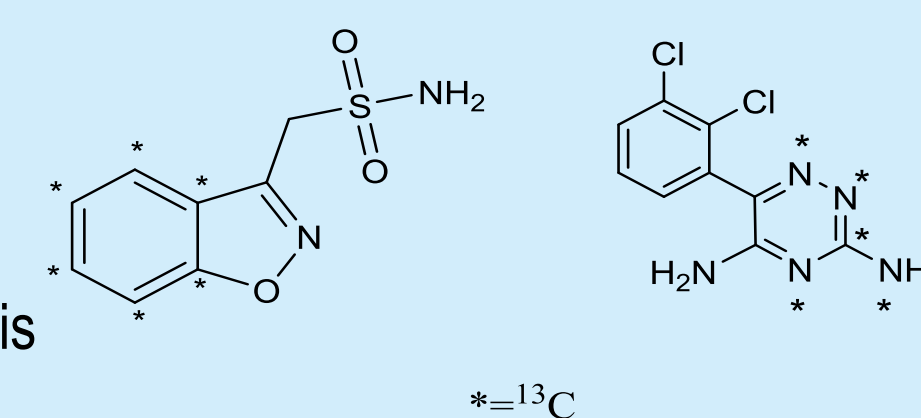
Both viable IS
End-user should select based on end use application

Testosterone-D₃ & Testosterone- $^{13}\text{C}_3$

- No deuterium scrambling at major transitions
- Major product ions of testosterone-D₃ are same as native - Requires optimization for low sensitivity applications
- Testosterone- $^{13}\text{C}_3$ provides greater sensitivity in low level testing (e.g. females)

Zonisamide- $^{13}\text{C}_3$, Lamotrigine- ^{13}C , $^{15}\text{N}_4$

- Structurally unsuitable for deuterium labeling:
- Protons susceptible to chemical exchange
- Potential H/D scrambling on aromatic ring during synthesis



Macromolecules, Peptides, Proteins

- Stable labeled internal standards for large bio-molecules are becoming more widely available. Peptides and oligonucleotides are produced by automated synthesizers using labeled starting materials. Internal standards of macromolecules and proteins have been produced by recombinant, fermentation, and semi-synthetic approaches which incorporate ^{13}C and ^{15}N building blocks into the biosynthetic process. Examples under development: stable labeled TG, IGF1, and Cyclosporin A

Cross Talk & Isobaric Interferences

Cross talk may be observed when:

- Analytes in the same panel are isobaric
- Impurities in the analyte are isobaric with the internal standard, or vice versa

Chromatographic resolution is required to mitigate cross talk from isobaric interferences and correction for contribution from isobaric compounds may be required

Example: Codeine and hydrocodone are isobaric. Impurities in the internal standard of one could produce an interfering signal at the retention time of the other impacting calibration results.

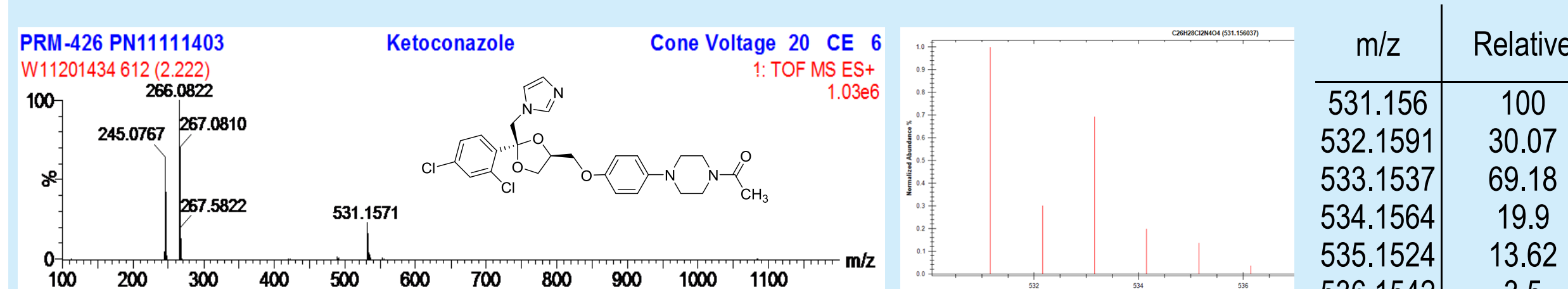
Natural Abundance & Isotope Distribution

- Some elements have a wider distribution of naturally occurring isotopes (e.g. Cl, Br, S)
- Improper mass selection due to relative abundance can lead to interferences between native and IS signals
- The actual isotope distribution and mass response of the M_n species depends on:
 - Isotopic distribution and purity of the available synthetic reagents
 - Chemical scrambling and exchange during manufacture
 - Contribution from naturally occurring isotopes of all elements in the molecule

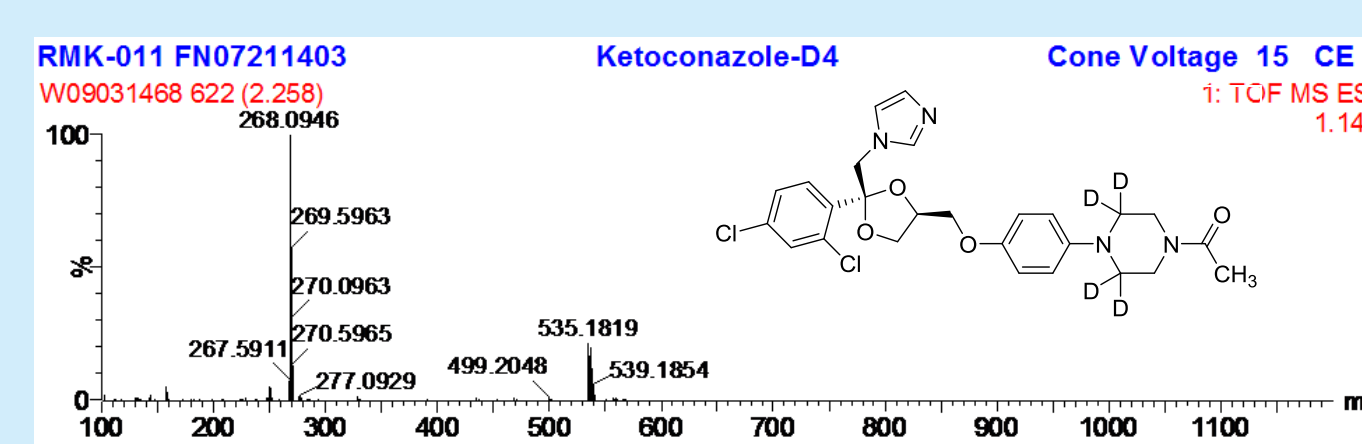
Element	Atomic Mass	Relative Abundance (%)
H	1	99.99
H	2	0.01
C	12	98.93
C	13	0.01
N	14	99.64
N	15	0.36
O	16	99.76
O	17	0.04
O	18	0.20
S	32	94.99
S	33	0.75
S	34	4.25
S	35	0.01
Cl	35	75.76
Cl	37	24.24
Br	79	51.00
Br	81	49.00

Example: Ketoconazole-D₄

- The highest abundance ($M+H$)⁺ ion of Ketoconazole-D₄ is m/z 535
- Natural isotopic distribution of native Ketoconazole will contribute ~13% to m/z 535 in the labeled IS trace
- Mitigate by monitoring the IS using m/z 537



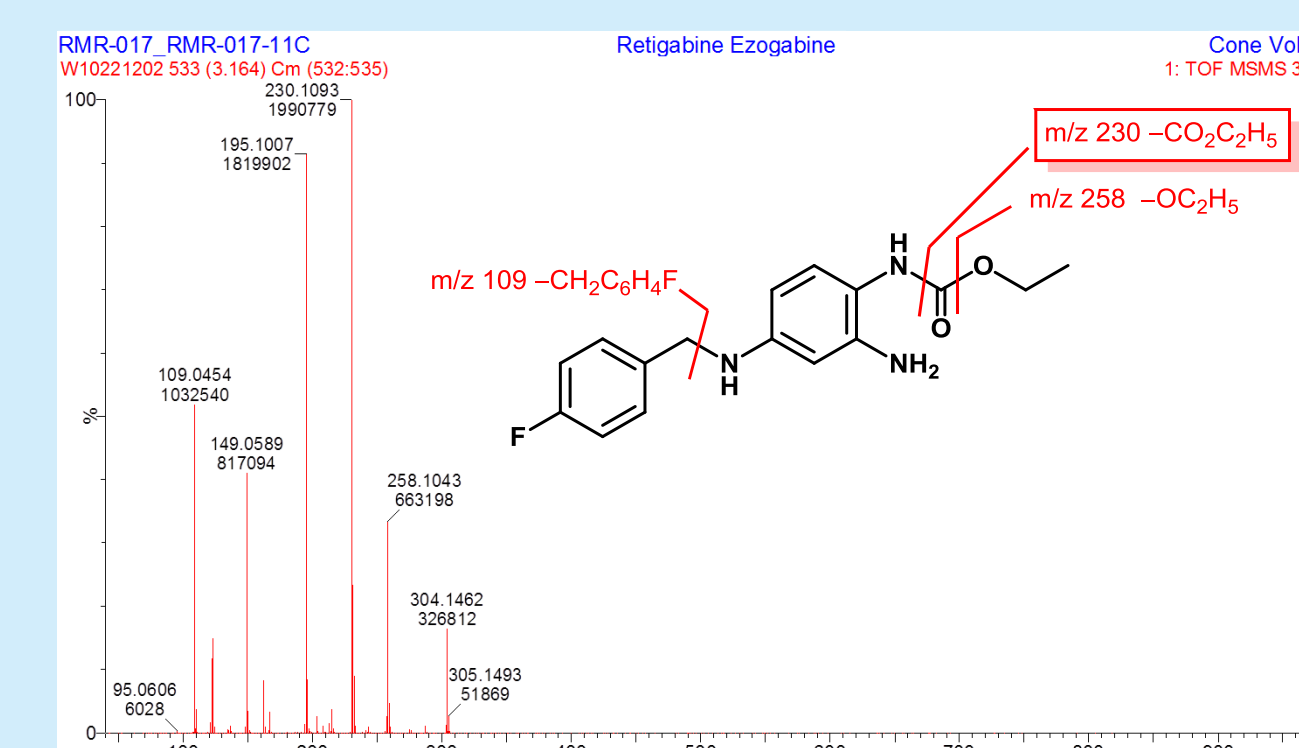
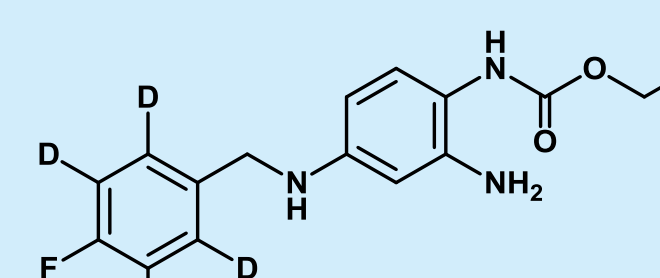
	Isotopic Distribution (%)	Adjusted Isotopic Distribution (%)
D ₀	0.01	0.01
D ₁	0.02	0.04
D ₂	0.12	0.24
D ₃	2.85	5.91
D ₄	29.64	60.47
D ₅	23.72	27.66
D ₆	27.07	5.67
D ₇	16.56	1.23



Label Position & MS Fragmentation

Suitability of label placement : Retention of label in the fragment

Example: Retigabine

Isotopic Distribution (%)*	
D ₀	0.011
D ₁	0.277
D ₂	6.593
D ₃	9.363
D ₄	82.800
D ₅	0.804
D ₆	0.153
D ₇ /D ₄	0.013%

Possible Label Positions:

- Simplest location is on the ethyl carbamate: OC₂D₅ or OCH₂CD₃ but unsuitable - Loss during MS fragmentation would occur
- Label on the central phenyl ring: daughter ion m/z 195, but unsuitable - High potential for exchange during synthesis
- Label on the 4-fluorophenyl-D₄ ring: daughter ion m/z 109 - Lower potential for scrambling or loss of D during synthesis

*Distribution reflects corrections for natural abundance of 18.8% M+1 and 2.01% M+2, M-1 and M-2 H radical loss observed in the native, and contributions from isotopic purity of reagent and scrambling during synthesis. Ratio of D₀/D₄ indicates there will be minimal interference with native analyte quantitation.

Internal Standards as Calibrators

- The concept of using an internal calibrator has been applied in other techniques such as quantitative NMR
- For LC-MS/MS applications this requires thorough understanding of the internal standard isotopic distribution pattern, correction for natural abundance, and impact of scrambling and cross-talk on isotope response. Platform specific variations in ionization efficiency and scrambling will also influence results
- Differences in ionization response between native and labeled IS must be well understood and may be dependent on platform, mode and instrument parameters
- Concentration of the internal standard must incorporate chromatographic purity, contributions from water, volatile and inorganic impurities, and concentration of the isotopic mass ion being used for quantitation

Label Position & H/D Scrambling

Chemical exchange and migration:

- Protons alpha to carbonyl systems are susceptible to chemical exchange and are not suitable for deuterium labeling. These labels may be susceptible to back exchange to H in solution.
- H/D exchange can also occur during catalyst mediated reactions during the synthetic process, and may be hard to control.

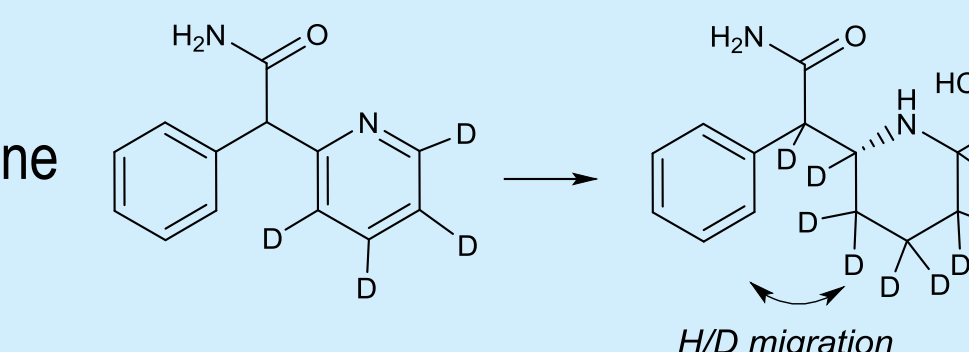
H/D exchange and scrambling in the MS:

- May occur in the LC-MS during collision or ionization
- Influenced by location of the deuterium relative to functional groups that can form resonance structures or cyclic charged species during ionization
- Mitigated through optimization of MS parameters and selection of transitions to monitor

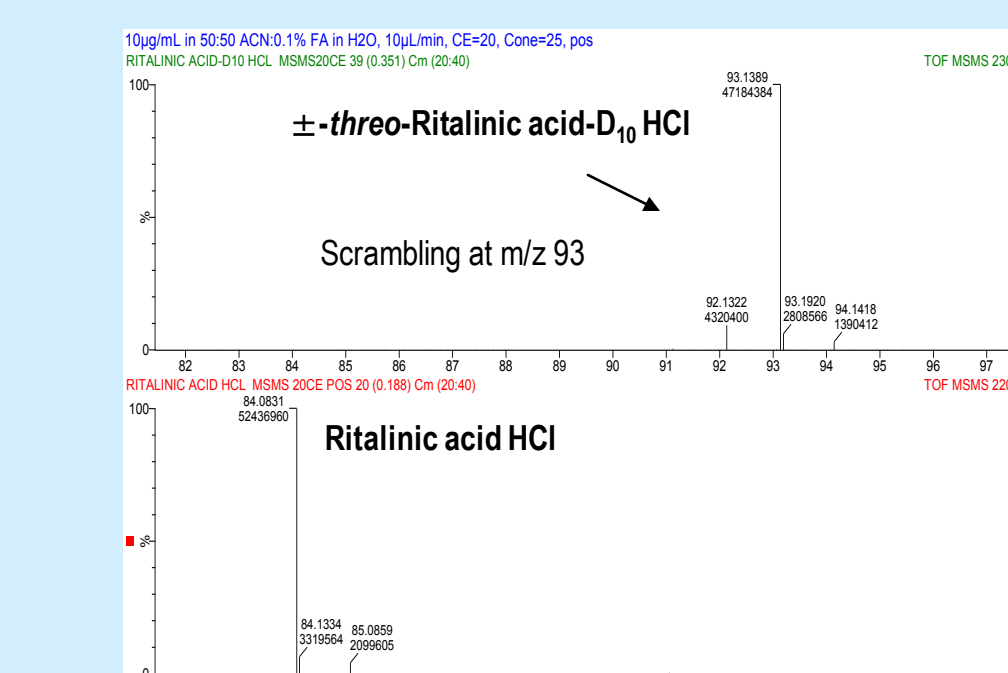
Example: Deuterated Ritalinic acid

Ritalinic acid-D₁₀

- Chemical exchange** of the alpha proton from H to D
- H/D migration** occurred during synthesis between the piperidine and aromatic rings (reduction of the pyridine to piperidine)
- QToF MS: ~55% D₁₀; 34% D₉; 9% D₈ and no D₀
- Quantitative NMR: ~ 5% H/D exchange between the aromatic and piperidine rings
- H/D scrambling was also observed during MS analysis** and mitigated through optimization of collision energy and resolution parameters:



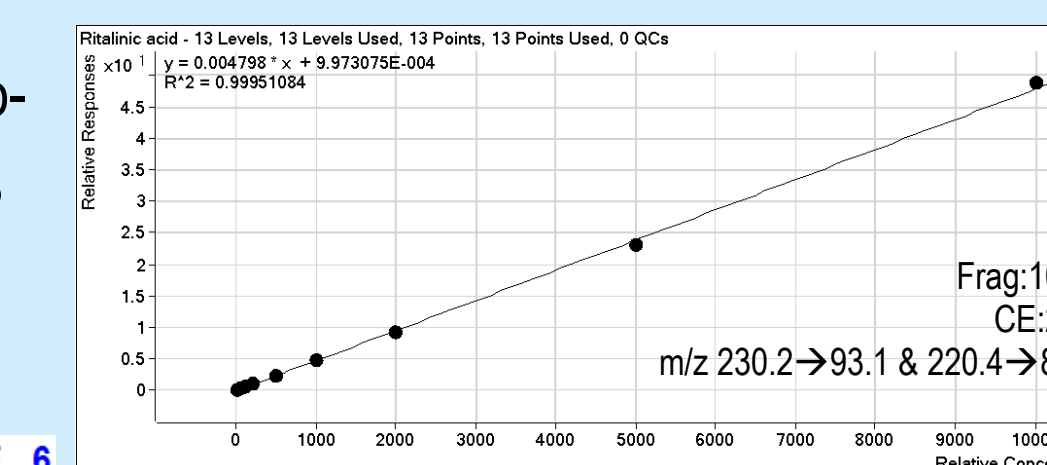
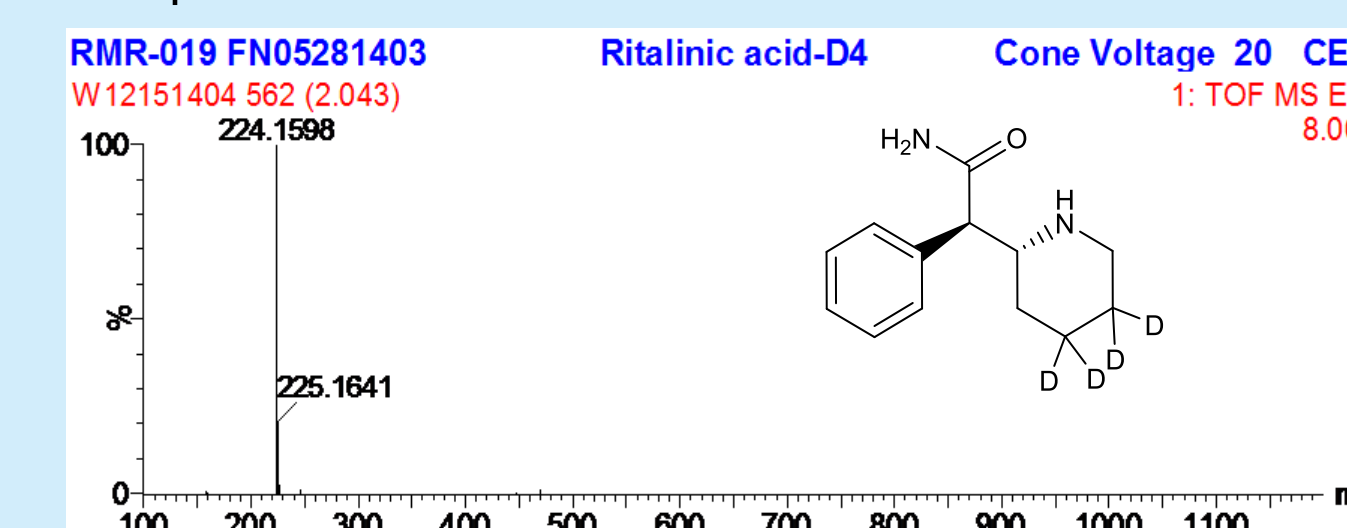
MS1 Resolution	Collision Energy	Label	Transition(s) dn	Scrambling % dn-1 / dn
Unit	20	D ₁₀ native	230.2→93.2 220.1→84.1	11.19 0.45
Wide	20	D ₁₀ native	230.2→93.2 220.1→84.1	11.31 0.46
Widest	20	D ₁₀ native	230.2→93.2 220.1→84.1	51.46 0.46



- Linearity achieved from 1 to 10,000 ng/mL
- Heavily deuterated internal standards can resolve chromatographically from the native, especially by UHPLC/MS

Ritalinic acid-D₄

- Developed as an alternative IS



Isotopic Distribution (%)	
D ₀	0.00
D ₁	0.00
D ₂	0.01
D ₃	1.48
D ₄	90.35
D ₅	8.16
D ₇ /D ₄	0.00%

Strategies for mitigation of scrambling

- Source exchange evaluation:
 - Infusion experiments compare mass distribution in first quadrupole of unlabeled molecule and labeled IS
 - Correct for natural abundance & isotopic purity. Corrected masses should differ by number of D atoms on IS
 - If no M_{n-1}, M_{n-2} detected, scrambling is occurring in the collision cell
- Evaluate effect of ionization mode, source voltages, temperature, gas flows and mass resolution on scrambling at different transitions
- Mitigation:
 - Adjust instrument parameters and collision energies to find the mass transition with the least scrambling
 - Evaluate interference from scrambling at LOQ
- If scrambling can not be adequately mitigated, consider a different IS

Summary

Selection of an appropriate internal standard depends on availability, cost, isotope effects/scrambling, method LOQ, and sensitivity requirements of the therapeutic monitoring range.

IS Comparison	^{13}C	D	Structural Analog
Normalize matrix effects		+++	++
Mass fragmentation	Similar to analyte; relative intensity of fragments may vary		Variable
Cost	Generally Higher	Lower	Low
Availability	Less	More	Wide
Chromatographic resolution from native	None	Highly deuterated IS (≥ 5) may separate	Usually separated from analyte
Chemical Exchange	No chemical exchange	H/D Exchange possible	May have different interactions in MS
Scrambling in the MS detector	None	Possible; Evaluate & control	Not applicable
Method Sensitivity	Optimal when low quantitation limits are required	Suitable for many therapeutic monitoring reference ranges	Depends on ability to control for matrix effects