Selection of Internal Standards for LC-MS/MS Applications

Uma Sreenivasan, Isil Dilek, Josh Cooper, Sarah Aijaz, Ravi Orugunty, Derrell Johnson, Heather Lima, Mitzi Rettinger, Cerilliant Corporation, 811 Paloma Dr Suite A, Round Rock, TX

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	 Ionization response and fragmentation pattern of IS similar to the analyte Adequate mass differentiation: IS MRM transitions should not interfere with analy MRMs
Chromatography	 Identical or close to analyte of interest for stable labeled analogs Chromatographically resolved from analyte for isobaric structural analogs
Purity	 Impurities do not cause interferences with analyte or other analytes in the test panel (ie. isobaric, co-eluting impurities) Isotopic purity: ratio of M₀/M_n Isotopic distribution : M₀M_n, M_{n+1}, M_{n+2} Isotopic distribution should be adjusted for natural abundance of isotopes (important for analytes with CI, 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	 Methods with wide dynamic range and/or high sensitivity (lower quantitation leve require internal standards that are high purity, co-elute with the analyte, and exhi 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), ¹³C or ¹⁵N 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, ¹³C, ¹⁵N ...

- · 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:

Testosterone-D₃ & **Testosterone-**¹³C₃

- No deuterium scrambling at major transitions
- Major product ions of testosterone-D₃ are same as native Requires optimization for low sensitivity applications

• Testosterone-¹³C₃ provides greater sensitivity in low level testing (e.g. females)

Zonisamide-¹³C₃, Lamotrigine-¹³C,¹⁵N₄

- 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 ¹³C and ¹⁵N 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.



End-user should select based on end use application



 $* = {}^{13}C$

	Natural Abundance & Isotope D	istri	but	ion
) ; ;)	 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 Mn species depends on: 	Element H C N	Atomic Mass 1 2 12 13 14 15	Relative Abundance (%) 99.99 0.01 98.93 0.01 99.64 0.36
	 Isotopic distribution and purity of the available synthetic reagents Chemical scrambling and exchange during manufacture 	0	16 17 18	99.76 0.04 0.20
:e	 Contribution from naturally occurring isotopes of all elements in the molecule 	S	32 33 34 35	94.99 0.75 4.25 0.01
	Example: Ketoconazole-D ₄	CI	35 37	75.76 24.24
	 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 	Br	79 81	51.00 49.00
	Mitigate by monitoring the IS using m/z 537 Natural Isotope	Abundar	nce of Ke	toconazole
	PRM-426 PN11111403 Ketoconazole Cone Voltage 20 CE 6 10 W11201434 612 (2.222) 1: TOF MS ES+ 10	C26H28CI2N4O4 (531.156037)	m/z	Relative
s) bit	$ \begin{array}{c} 100 \\ 245.0767 \\ 267.0810 \\ 0 \\ 100 \\ 200 \\ 300 \\ 400 \\ 500 \\ 600 \\ 700 \\ 800 \\ 900 \\ 100 \\ 10$		531.1 532.18 533.18 534.18 535.18 536.18	5610059130.0753769.1856419.952413.625423.5

	Isotopic	Adjusted Isotopic	
	Distribution (%)	Distribution (%)	
D_0	0.01	0.01	RMK-011 FN07211403 Ketoconazole-D4
D_1	0.02	0.04	W09031468 622 (2.258) 100 268.0946
D_2	0.12	0.24	
D_3	2.85	5.91	269.5963 CI
D_4	29.64	60.47	
D_5	23.72	27.66	267.5911 277.0929 499.2048 539.1854
D_6	27.07	5.67	0- <u>++++++++++++++++++++++++++++++++++++</u>
D ₇	16.56	1.23	

Label Position & MS Fragmentation

Suitability of label placement : Retention of label in the fragment **Example: Retigabine**



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 D_0/D_4 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

test

JITIDIE

on level nd exhi



Cone Voltage 15 CE 1: TOF MS ES+



0.011 0.277 6.593 9.363 82.800

0.804 0.153

0.013%

 D_0/D_4

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

H/D scrambling was also observed during MS analysis and mitigated t energy and resolution parameters:

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



• Heavily deuterated internal standards can resolve chromatographically from the native, especially by UHPLC/MS **Ritalinic acid-D**₄

nic acid - 13 Levels. 13 Levels l

 Developed as an alternative IS Cone Voltage 20 CE 6 Ritalinic acid-D4 1: TOF MS ES+ W12151404 562 (2.043) 8.00e5

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 ef- fects	++++ ++								
Mass fragmentation	Similar to analyte; relativ	Variable							
Cost	Generally Higher	Lower	Low						
Availability	Less	More	Wide						
Chromatographic resolution from native	None	Usually separated from analyte							
Chemical Exchange	No chemical exchange	May have different in- teractions in MS							
Scrambling in the MS detector	None	Not applicable							
Method SensitivityOptimal when low quantitation limits are requiredSuitable for many therapeutic monitoring reference rangesDepends on ability to control for matrix ef- fects									

Isotopic Distribution (%)						
D ₀	0.00					
D1	0.00					
D ₂	0.01					
D ₃	1.48					
D ₄	90.35					
D ₅	8.16					
D_0/D_4	0.00%					

85.08	859													
2099	605												— m/z	
5	86	87	88	89	90	91	92	93	94	95	96	97		
d, 1	13 Po	ints, 13	Points I	Jsed, (QCs									
6E-0	04							_				-		_
_								_						
_										/	-			_
_									\square					_
_														_
_														
_					•						Ero	a.10	າງາ	
_											гіа	g. It	JZV	
	/				_							CE:2	20V	
T					m	\/ 7 '	230 2	<u>د</u> ر	93 1	8.2	20 /	ک د	A 1	
					- 11	" <i>∠</i> '	200.2	- /	55.1	αz	20.4		ו.די	
														_
ooo		3000	4000	5	000	600	0 7	000	80'08) 9	000	1000	0	

ine rings d through optimiz	ation	of collision
A in H2O, 10µL/min, CE=20, Cone=25, pos 20CE 39 (0.351) Cm (20:40)	02 1290	TOF MSMS 230.50ES+
- <i>threo</i> -Ritalinic acid-D ₁₀ HCI	47184384	4.1201
Scrambling at m/z 93		

82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 INIC ACID HCL MSMS 20CE POS 20 (0.188) Cm (20:40)

Ritalinic acid HCI

92.1322 4320400 93.1920 2808566 1300412

a SIGMA-ALDRICH[®] company