EVERYTHING YOU WANTED TO KNOW ABOUT INTERNAL STANDARDS, BUT WERE TOO AFRAID TO ASK

PART 1: WHAT, WHY AND HOW

RUSSELL GRANT

LABORATORY CORPORATION OF AMERICA® HOLDINGS, BURLINGTON, NC USA
Disclosures

- None
Analyte must have identical matrix effects and extraction efficiency (i.e., total efficiency) between calibrators and samples for accuracy
External Calibration cannot solve recovery differences

Hydrolysis (100% Efficiency)

Hydrolysis (50% Efficiency)

-50% Bias
An internal standard in **analytical chemistry** is a **chemical substance** that is added in a constant amount to samples, the blank and **calibration** standards in a **chemical analysis**.

Used to correct for the loss of analyte during sample preparation, injection and ionization.
When should IS be added?

1. Thaw and mix Calibrators/QC’s/Specimens
2. Addition of Internal Standard
3. Extraction/Purification
4. Liquid Chromatography
5. Interface
6. Precursor Ion Selection
7. Fragmentation
8. Product Ion Selection
9. Detection

- **Ensure Homogeneity**
- **Recovery/Sample variance Correction**
- **Reduce Complexity, remove interferences**
- **Gas phase ion cluster generation**
- **Tandem MS Selectivity step 1**
- **Reproducible fragmentation to generate structurally unique motif**
- **Tandem MS Selectivity step 2**
- **Ion counting/signal amplification**
Difference in the matrix effects and extraction efficiency (total efficiency) incurred by the **Analyte** between calibrators and samples should be identical to the difference incurred by the **Internal Standard**.
External Calibration with Poor Internal Standardization

**Hydrolysis**

- 100% Efficiency
- 50% Efficiency

**CALIBRATORS**

**SAMPLES**

+ Labeled-IS

Bias: 50%
External Calibration with Good Internal Standardization

CALIBRATORS

+Labeled-IS

Hydrolysis (100% Efficiency)

SAMPLES

Hydrolysis (50% Efficiency)

0% Bias
What is a Good IS?...pick your favorite

Testosterone D2
$m/z$ 291 – 99, 111

Testosterone D3
$m/z$ 292 – 97, 109

Testosterone D5
$m/z$ 294 – 100, 111

Testosterone D5
$m/z$ 294 – 102, 114

And now?
$^{13}C_{13}$ Testosterone
$m/z$ 292 – 100, 112

I “guessed” at transitions for the second D5 IS - H/D Scrambling?
Considerations for Degree of Labelling – Natural abundance

<table>
<thead>
<tr>
<th>Element</th>
<th>Atomic Mass</th>
<th>Relative Abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>1</td>
<td>99.99</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.01</td>
</tr>
<tr>
<td>C</td>
<td>12</td>
<td>98.93</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>1.07</td>
</tr>
<tr>
<td>N</td>
<td>14</td>
<td>99.64</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.36</td>
</tr>
<tr>
<td>O</td>
<td>16</td>
<td>99.76</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.20</td>
</tr>
<tr>
<td>S</td>
<td>32</td>
<td>94.99</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>4.25</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>0.01</td>
</tr>
<tr>
<td>Cl</td>
<td>35</td>
<td>75.76</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>24.24</td>
</tr>
<tr>
<td>Br</td>
<td>79</td>
<td>51.00</td>
</tr>
<tr>
<td></td>
<td>81</td>
<td>49.00</td>
</tr>
</tbody>
</table>

Deuterons and Isotopes | m/z | Relative Abundance Ketoconazole | Isotopic Distribution D₄-Ketoconazole |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>531.156</td>
<td>100</td>
<td>0.01</td>
</tr>
<tr>
<td>1</td>
<td>532.159</td>
<td>30</td>
<td>0.04</td>
</tr>
<tr>
<td>2</td>
<td>533.154</td>
<td>69</td>
<td>0.24</td>
</tr>
<tr>
<td>3</td>
<td>534.156</td>
<td>20</td>
<td>5.91</td>
</tr>
<tr>
<td>4</td>
<td>535.152</td>
<td>14</td>
<td>60.47</td>
</tr>
<tr>
<td>5</td>
<td>536.154</td>
<td>4</td>
<td>27.66</td>
</tr>
<tr>
<td>6</td>
<td>537.156</td>
<td>0</td>
<td>5.67</td>
</tr>
<tr>
<td>7</td>
<td>538.158</td>
<td>0</td>
<td>1.23</td>
</tr>
</tbody>
</table>

“Walk the Isotopes” and use m/z 537 for D₄-IS

Courtesy of Mitzi Rettinger, MilliporeSigma
Considerations for Position of Labelling

Labelling positions:

- O\textsubscript{C\textsubscript{2}}D\textsubscript{5} or OCH\textsubscript{2}CD\textsubscript{3} - loss during MS fragmentation?
- Central phenyl ring - H/D exchange during synthesis?
- 4-fluorophenyl ring - Lower scrambling, H/D exchange

<table>
<thead>
<tr>
<th>Isotopic Distribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D\textsubscript{0}</td>
</tr>
<tr>
<td>D\textsubscript{1}</td>
</tr>
<tr>
<td>D\textsubscript{2}</td>
</tr>
<tr>
<td>D\textsubscript{3}</td>
</tr>
<tr>
<td>D\textsubscript{4}</td>
</tr>
<tr>
<td>D\textsubscript{5}</td>
</tr>
<tr>
<td>D\textsubscript{6}</td>
</tr>
</tbody>
</table>

D\textsubscript{0}/D\textsubscript{4} ratio – adequate selectivity

Selection of Internal Standards for LC-MS-MS
Structurally Unique in specimens and between each other.

- **Methionine**
  - (m/z): 150 – 104
  - Tr = 12.57

- **D₃-Glutamic Acid**
  - (m/z): 153 – 88
  - Tr = 13.68

- **Valine**
  - (m/z): 118-72
  - Tr = 12.89

- **D₃-Proline**
  - (m/z): 119-73
  - Tr = 13.87

- **D₃-Lysine**
  - (m/z): 150 - 87
  - Tr = 19.03

- **D₃-Methionine**
  - (m/z): 153 – 107
  - Tr = 12.57
What do you think about the * peak at 1 minute for HVA?

Now add $^{6}C_{13}^{18}O$ HVA IS

How about now?

Now add Qualifier Transition

Transition ratio Agreement 98%
Using IS to inform Peak Picking parameters

Analyst: Auto Integration

Analyst: Manual integration

Ascent: Automatic optimization of quantitation parameters

Manual adjustment of parameters is labor intensive and introduces human bias
Set relative agreement (Analyte and IS) in integration methods “narrowly”
What Does the IS Tell You Qualitatively

**Qc1 (injection #12)**

- Retinol
- D₄ Retinol

**Qc1 (injection #94)**

- Retinol
- D₄ Retinol

Good IS informs analyte retention time and peak shape

Enables diagnosis of problems – guides correction
How much IS should you add?

How much? 100-fold range – Mid point, 1000-fold range 10 – 25 x LLOQ
How should you add IS? – be precise, precisely!

Reverse T3 Manual SPE
First time ever

Reverse T3 Automated SPE
First time ever

D₅ Phenylalanine trend – second LC channel started (different solvent chemistry)

Calibrators re-injected = System drift (not prep)

Addition and/or Recovery Variance of IS makes outlier detection Impossible
Tecan Liquid handler IS addition
Using same 8 tips with aqueous D₄-Cortisol Drift across run observed

With 0.1% BSA (aq) D₄-Cortisol solution and Pre-wetted tips (x3) prior to dispensing
IS peak area CV = 8.6%

IS peak area precision enables outlier detection
How should you add IS? Protein Precipitation issues...

Acetone, Acetonitrile, Ethanol, Isopropanol, Methanol

Precipitation techniques are FAST, Q: IS recovery same as analyte?
Aliquots of sample pool equilibrated with IS for decreasing amounts of time prior to extraction in parallel (i.e., reverse timing).

- 60 min
- 30 min
- 15 min
- 5 min

How to evaluate IS addition?

Plateau in A:IS ratio indicates IS has reached binding equilibrium with analyte. Use double the time.
Vitamin B2 Flavin Adenine Dinucleotide in Whole Blood

Flavine Adenine Dinucleotide

Flavine Adenine Dinucleotide $^{13}$C$_5$ Ammonium Salt

Calculate as Fractions (recovery) relative to max/min mixing time

Use Individual samples and interrogate IS peak area trend too!
WHAT Internal Standards should be

NATURE:

- Structurally unique (exogenous) – *Not observed in samples*
- Resolved (separated) from analyte(s) by MS, *but Co-elute*
  - *Stable labeled isotope* ($^{13}$C, $^{15}$N, $^{18}$O, $^2$H *in order*) $> +3$ amu
- No H/D Exchange – *check stability in solution and ion source*
- Structurally similar (analog) – *Limited recovery or ionization effect correction*
- Structurally dissimilar - *injection check at best?*
WHY Internal Standards are used

UTILITY:

- Identification of analyte retention time shift and peak shape
- Injection variance
- Normalization of recovery differences
- Normalization of ionization effects between calibrators and samples
- *One of the most valuable components in LC-MS/MS analytical quality*
HOW Internal Standard should be used

**ADDITION:**

- FIRST step after mixing/pipetting sample (*Identical biochemically to analyte*)
- Reproducibly added (*precise!*!) to samples, calibrators, QC’s except double blanks
- Solution ideally miscible with sample – *to correct analyte recovery if equilibrium is a concern*
- Mixed well prior to extraction – *Equilibrated identically to analyte – you should always prove this with real samples versus calibrators*
EVERYTHING YOU WANTED TO KNOW ABOUT INTERNAL STANDARDS, BUT WERE TOO AFRAID TO ASK

PART 2: BUT WHAT ABOUT WHEN....

RUSSELL GRANT

LABORATORY CORPORATION OF AMERICA® HOLDINGS, BURLINGTON, NC USA
Analyte observed in IS solution

**IS in Water APCI Source**

D₄-Dopamine IS
1e5 cps

Dopamine
2e6 cps

**IS in Water ESI source**

D₄-Dopamine IS
4e5 cps

**Neat Dopamine D₄ solution appeared to contain mostly Dopamine**

Labelling position is important - ESI confirmed purity and used for assay
LAST ON – FIRST OFF!
**Ion Sources do weird things**

- **Q1 Scan APCI +ve ion**
- **Q1 Scan APCI -ve ion**

**Radical Cation and isotopic variance leads to measurement variance**

**IS imprecision in calibrators and samples should guide Ion Source conditions**

**D₆ α-Tocopherol Imprecision**
- APCI -ve CV = 7%
- APCI +ve CV = 32%

**m/z, Da**
- 434, 435, 436, 437, 438, 439

**[M+H]⁺ and [\(^{13}\text{CM}\)+]**

**[M⁻]**

**[\(^{13}\text{CM}+\text{H}⁻\)+]**

**[M+H⁻₂]⁺**

**[M⁺]**

**[\(^{13}\text{CM}+\text{H}⁺\)+]**

**Index**
- NO D₆ α-Tocopherol

**IS Peak Area, counts**
- 1.0e8, 2.0e8, 3.0e8

**NO D₆ α-Tocopherol**

- m/z, Da: 433, 434, 435, 436, 437, 438

**Imprecision**
- APCI -ve CV = 7%
- APCI +ve CV = 32%
Curves Diverge?

Chlorpromazine
\( m/z \ 319-86 \)

D3-Chlorpromazine
\( m/z \ 322-89 \)

1\textsuperscript{st} (top) verses 8\textsuperscript{th} (bottom) divergence using “matched” transitions

Analyte Area / IS Area

<table>
<thead>
<tr>
<th>Analyte Conc. / IS Conc.</th>
<th>0</th>
<th>600</th>
<th>1200</th>
<th>1800</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyte Area / IS Area</td>
<td>0</td>
<td>8</td>
<td>16</td>
<td>32</td>
</tr>
</tbody>
</table>
Facile Fragmentation

Repeat injection of a Known sample
Starting with a Cold ion source

Product Ion retains N-dimethyl functionality (Deuterons)

1st verses 8th Curves using “mis-matched” transitions

Chl: 319/86, IS 322/89
Chl: 319/246, IS: 322/89

Accuracy (%) vs Injection Number

IntENSITY (cps)

0.0 100 200 300 m/z, Da

0.0 4.0e7 8.0e7 1.2e8 1.6e8

Analyte Area / IS Area

60 70 80 90 100 110 120 130 140

6 8 10 12 14

Analyte Conc. / IS Conc.
Transition Selection to minimize Isobaric Contribution

>20 transitions screened per isobaric pair in matrix samples, not just neats

Most sensitive ≠ best transition to use
More Isobaric (and Isotopic) Hindrances – selectivity!

**Hydrocodone**

m/z = 300.2 → 257.2

**Codeine**

m/z = 300.2 → 215.2

**Hydromorphone**

m/z = 286.2 → 185.2

**Morphine**

m/z = 286.2 → 155.2

**Oxymorphone**

m/z = 302.2 → 242.2

**Dihydrocodeine**

m/z = 302.2 → 245.2

- **Hydrocodone-d₃**
  - m/z = 303.2 → 241.2
- **Codeine-d₆**
  - m/z = 306.2 → 115.2
- **Hydromorphone-d₆**
  - m/z = 292.2 → 185.2
- **Morphine-d₃**
  - m/z = 289.2 → 152.2
- **Oxymorphone-d₃**
  - m/z = 305.2 → 230.2
- **Dihydrocodeine-d₆**
  - m/z = 308.2 → 230.2

- **D₃** or **D₆** IS’s in isobaric pairs for automated peak selection
- Mismatched IS transitions for peak purity

- **D₆- Codeine** because of $^{13}$C contribution of Oxym/DHC

4 analytes and 4 IS’s in 8 mass unit range
Too many Deuterons can hurt you

**Fast LC: Co-elution**

**Slow LC: Separation**

**1:1 Gabapentin:D10 IS in Clean Urine**

\[ \text{Mean} = 100\% \]

**D10-Gabapentin IS not Co-eluting**

\[ \text{Mean} = 92\% \]

**Co-elution is VERY important**

Courtesy of Brian Rappold, ASMS poster 2012 – Deception in the Deuteriums
Too few labels can hurt you also

Calibration curve non-linear 3-log range for 11-Desoxycortisol

$^{13}C_2$ labelled IS only (*)

Analyte isotopically contributes to IS

Solutions:

Truncate to linear range assay neat and pre-dilute samples (two analysis!),

Repeat on dilution > mid point

Increase concentration/alternate IS

Clinically acceptable as is
IS response decreases in Calibrators + Quadratic Curve

Multiplex assay 25000-fold range

- Methylphenidate (1-250 ng/mL, D$_9$ IS),
- 9-OH Risperidone (1-250 ng/mL, D$_4$ IS),
- Risperidone (1-250 ng/mL, D$_4$ IS),
- Chlorpromazine (10-2500 ng/mL, D$_3$ IS)
- Haloperidol (1-250 ng/mL, D$_4$ IS),
- Fluphenazine (0.1-25 ng/mL, D$_8$ IS*)

IS response versus $3^{rd}$ Calibrator with increased analyte concentrations

Most show some reduction in signal
Does it result in error?

Risperidone and 9-OH Risperidone
IS response reduction and quadratic curve

Detuned MASS Spectrometer Transition
efficiency for Analyte Transitions ONLY

*Culprit was Multiplier Blinding as source suppression affects BOTH Analyte and IS*

Are you observing it co-suppression or detector blinding
Dynamic Extraction with PPT plates

Testosterone $+^{13}C_3$ Testosterone in Methanol
Add IS to plate, then add Serum, mix 5 min, vacuum

Dynamic (non-equilibrated) extraction obvious in charcoal stripped serum calibration matrix

Off line mixing, centrifugation then addition to PPT plate OK (ish)
Extraction: Increased Recovery or reduced Imprecision?

Go for reduced imprecision of IS every time

Enables outlier detection!
Estradiol bias for Calibrators, QC pools/Samples when mixing prior to SLE

Do we believe the MS results? YES

Consistent across matrix types? YES

Timing of the experiment (temperature of samples/pipetting consistency?) YES

High quality IS? $^{13}$C$_6$ for BOTH

Observed in predicate LLE assay? NO..it was a 90 minute mixing step

Solution: Evaluate Equilibration of Analyte and IS
Temperature affects binding kinetics...obviously.

Mixing at 37°C – equivalent accuracy (analyte/IS) recovery over time

**Note:** This informs how you perform Spike and Recovery in validation
Perfectly labelled Internal standard added in Methanol (PPT/Mix)

Riboflavin

$^{13}C_4^2N_{15}$ Riboflavin

Riboflavin recovery ratio in Plasma

$^{13}C_4^2N_{15}$ Riboflavin recovery over time
Reached equilibrium yet? Incomplete recovery?

**Riboflavin recovery ratio in Plasma 37 °C**

**Riboflavin recovery ratio in Plasma + 0.1% Formic Acid**

**FAD-IS recovery in Plasma 37 °C**

**VitB2 RBC Biology**

**VitB2 Plasma Biology**

Riboflavin

Flavin Mononucleotide

Flavin Adenine Dinucleotide

Fast in plasma

Slow in plasma

$\text{Riboflavin}$

$\text{Flavin Mononucleotide}$

$\text{Flavin Adenine Dinucleotide}$
Oddly...Riboflavin unchanged up to 4 hours following thaw in plasma samples
EVERYTHING YOU WANTED TO KNOW ABOUT INTERNAL STANDARDS, BUT WERE TOO AFRAID TO ASK

PART 3: UNIQUE CAPABILITIES

RUSSELL GRANT

LABORATORY CORPORATION OF AMERICA® HOLDINGS, BURLINGTON, NC USA
Release? Reinject or Re-extract?

What is the IS correcting for
Recovery
Transfer
Injection
Ionization?

<table>
<thead>
<tr>
<th>Index</th>
<th>IS Peak Area, counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

Mix, Equilibrate, SLE, Evaporate

Mix, Transfer, LC-MS/MS

Testosterone
m/z 289 – 97, 109

$^{3}\text{C}_{13}$ Testosterone
m/z 292 – 100, 112

Recon with different IS
D$_5$-Testosterone
m/z 294 – 100, 111
Two IS’s is better than one

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume Added to SLE (%)</td>
<td>100</td>
<td>75</td>
<td>50</td>
<td>25</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Volume Injected (%)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>75</td>
<td>50</td>
<td>25</td>
</tr>
</tbody>
</table>

- **Testosterone**
  - $^3C_{13}$ Testosterone

- **$^{12}D_5$-Testosterone**
  - $^3C_{13}$ Testosterone
And when tested experimentally....

| Testosterone/$^{13}$C$_3$ Testosterone Ratio | 1.2 | 1.2 | 1.1 | 1.1 |
| D$_5$ Testosterone/$^{13}$C$_3$ Testosterone Ratio | 1.7 | 2.1 | 3.3 | 6.8 |

**SLE Under delivery: Unless you REALLY know of volumetric errors – Re-Extract**

| Testosterone/$^{13}$C$_3$ Testosterone Ratio | 1.2 | 1.2 | 1.1 | 1.1 |
| D$_5$ Testosterone/$^{13}$C$_3$ Testosterone Ratio | 1.7 | 1.7 | 1.6 | 1.5 |

**Injection/Ionization error: Release if responses acceptable or re-inject**
Using “bracketing” calibration curves...you are averaging drift across a run

Using a single curve...you are performing Historical Calibration

....because the IS enables you to do so

Figures of merit for Inter-assay (n=20 at 6 levels)

Levetiracetam: Imprecision < 10.07%, Bias < 6.35%

Oxcarbazepine: Imprecision < 6.03%, Bias < 9.98%

Lacosamide: Imprecision < 8.58%, Bias < 6.03%
The IS actually enables Historical calibration

Freeze separate aliquots, thaw a single aliquot and assay (Day zero)
Store working IS solution in stable conditions (solution, container, temperature)

24 hours later...thaw another aliquot and use stored IS –
No calibrators – measure Analyte/IS ratio against day zero curve
Compare Day zero (x) to + 24 hours (y)

Levetiracetam + 24 hours
Deming Slope = 1.016
Intercept = -0.7749
Corr Coef, r = 0.9955

Oxcarbazepine + 24 hours
Deming Slope = 1.039
Intercept = -0.8327
Corr Coef, r = 0.9956

Lacosamide + 24 hours
Deming Slope = 1.039
Intercept = -0.9056
Corr Coef, r = 0.9927
7 days later

Levetiracetam + 144 hours
Deming Slope = 1.217
IS Degraded - only good for 3 days
Intercept = -1.1966
Corr Coef, $r = 0.9892$

Oxcarbazepine + 144 hours
Deming Slope = 1.041
Intercept = -0.7982
Corr Coef, $r = 0.9927$

Lacosamide + 144 hours
Deming Slope = 1.065
Intercept = -0.8589
Corr Coef, $r = 0.9920$
LC-MS/MS (FDA Trial samples) versus TFC-LC-MS/MS

When sample preparation is painful

TFC-LC-MS/MS – pH Modification required

Total Dabigatran (Including Glucoronide)

pH 11, 2.5 Hr @ 37°C = 182.484 ng/mL

Free Dabigatran (Unconjugated)

pH 3 = 105.470 ng/mL

LC-MS/MS (FDA Trial samples) versus TFC-LC-MS/MS

Scatter Plot

---

<table>
<thead>
<tr>
<th></th>
<th>Deming</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope</td>
<td>0.951 (0.930 to 0.973)</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.6182 (-3.0005 to 4.2369)</td>
</tr>
<tr>
<td>Std Err Est:</td>
<td>8.7582</td>
</tr>
</tbody>
</table>

Corr Coef (R): 0.9959
Bias: -0.0322
X Mean ± SD: 136.5904 ± 100.7016
Y Mean ± SD: 130.5582 ± 95.8182
Std Dev Diffs: 10.1615
For samples > ULOQ (Calibrated Analyte peak area)

Dilute then add IS

5000ng/mL (5 x ULOQ)
CV = 7.52%
Bias = 12.11%

BUT...Look closely at the Axis

Add IS here!

For Linear-fit calibration curves: (y=mX-b)

Dilute “in-well” and re-inject or inject less so that

Analyte peak area response ≤ ULOQ (linear range)
IS peak area response > Analyte LLOQ (ideally 10x)

Linear calibration fit = linear response of analyte - applies to good IS’s, even lower “amounts”

Reduced bias observed as only 1 step pre IS not 4
Now you are thinking about calibration differently... RMP’s

Step 1: Analyze samples using external calibration and isotope dilution

- Analyte peak area response (Ar)
- IS peak Area response (Ir)

Analyte peak area response (Ar) / Internal standard concentration (Ic)

Step 2: Determine Equimolar relationship between Analyte and IS responses

1.1% Carbon is in the form of $^{13}$C, Per NIST:

- Testosterone ($^{13}$C$_{19}$H$_{28}$O$_2$) isotope ratio’s 77%, 21%, 2%
- $^{13}$C$_3$ Testosterone ($^{13}$C$_3$-$^{12}$C$_{16}$H$_{28}$O$_2$) isotope ratio’s 80%, 18%, 2%

Difference in Isotopic Contribution = 3%

100µM $^{13}$C$_3$ Testosterone = 103µM Testosterone
Bracketing or Absolute Matching with IS

Step 3: Analyze using “corrected” response function with Bracketing IS concentration

\[
[\text{Analyte}] = \frac{\text{Analyte Area}}{\text{IS Area}} \times \text{IS } \]

Left: \( \frac{100000}{90000} \times 500 \text{ ng/dL} = 555 \text{ ng/dL} \)

Right: \( \frac{100000}{115000} \times 600 \text{ ng/dL} = 521 \text{ ng/dL} \)

Average = 538 ng/dL

Or repeat analysis with IS modification until signal responses match \([\text{A}] = [\text{IS}]\)

\([\text{IS}] = 520 \text{ ng/dL} < [\text{A}]\)

\([\text{IS}] = 530 \text{ ng/dL} < [\text{A}]\)

\([\text{IS}] = 540 \text{ ng/dL} = [\text{A}]\)
Now you are really thinking about calibration differently.
How about no external calibration whatsoever? Instrinsix ®

Protein precipitation workflow

Addition of differentially labelled C\textsubscript{13} Methotrexate Calibrators
Known amounts added
No contribution between or to Methotrexate
13C has no effect on retention time

\begin{align*}
\text{Methotrexate Calibrator 1} & : C_9^{13C_{11}}H_{22}N_8O_5 (0.025 \, \mu M) \\
\text{Methotrexate Calibrator 2} & : C_{15}^{13C_5}H_{22}N_8O_5 (0.1 \, \mu M) \\
\text{Methotrexate Calibrator 3} & : C_{14}^{13C_6}H_{22}N_8O_5 (1.0 \, \mu M) \\
\text{Methotrexate Calibrator 4} & : C_6^{13C_{14}}H_{22}N_8O_5 (10 \, \mu M)
\end{align*}
Intrinsix® = Internal Calibration with an IS curve per sample

Calibrator 4
\( C_6^{13}C_{14}H_{22}N_8O_5 \) (10 µM)

Calibrator 3
\( C_{14}^{13}C_6H_{22}N_8O_5 \) (1.0 µM)

Calibrator 2
\( C_{15}^{13}C_5H_{22}N_8O_5 \) (0.1 µM)

Calibrator 1
\( C_9^{13}C_{11}H_{22}N_8O_5 \) (0.025 µM)

Methotrexate
\( C_{21}H_{22}N_8O_5 \) ?

Measured = 2.14 µM (bias < 7.5%)

1/x weighting
\( r^2 = 0.999813 \)
QA materials from UK NEQAS (pilot scheme) and WEQAS
Correlation between IntrinsiX and conventional UPLC-MS/MS analysis described by Deming equation $y = -0.99x - 0.02$ ($n=23$, range 0.025-2.18 µmol/L)
Q: Internal Calibration versus External calibration and Internal Standardization?

3 different Full-length NAT-Tg

Full-length SIL-Tg

3 pmol

8 different cleavable SIL (cSIL) peptides

8 different fully-tryptic SIL (tSIL) peptides

*3 pmol (each)

*1.5 pmol (each)

0.1% HSA

30 min, 56 °C
10 mM DTT +Denaturant

30 min, 37 °C
1:10 Substrate:Enzyme
TPCK-treated Bovine Trypsin

LC-SRM

Go Big or Go Home

Internal Calibration

Absolute Tg Measure (pmol)

- Under-recovery: Digestion Efficiency of SIL > Analyte
- Over-recovery: Digestion Efficiency of SIL < Analyte
- Isotope Effect?
External Calibration with Recombinant Protein and Internal standardization

TFP peptide generation in Recombinant Tg > Human Tg

I hope I passed the audition...Questions?
Slide 1: Hello
Slide 2: No disclosures, except I do like Guinness...particularly if you are buying
Slide 3: External calibrators are used to generate a dose dependent response when used in the assay – i.e. generating a Calibration curve. Analyte recovery and matrix effects (total efficiency) must be identical between calibrators and samples for accurate measurement of analyte in unknown samples
Slide 4: In this example, hydrolysis of the glucoronide to the parent molecule is complete in calibrators but incomplete in an unknown samples resulting in a negative bias.
Slide 5: Perfect Internal Standards behave identically to the analyte – both physicochemically and analytically – Stable labeled forms are the go to choice!
Slide 6: Internal standards only correct steps following addition...if used properly!
Slide 7: Addition of a stable labelled version of the analyte – the IS - to calibrators and samples prior to extraction, separation and ionization. Differences in the analyte are exhibited by the IS, thus the IS serves corrects efficiency losses between the calibrators and samples, minimizing bias. A properly selected IS fixes a lot of problems and no other technology has this powerful tool.
Slide 8: IS added after hydrolysis can correct for differences in injection volume, chromatography retention time variance and ionization differences – but – does not correct for the preparation efficiency difference between calibrators and samples, Bias still present.
Slide 9: The Internal standard is added prior to hydrolysis for both calibrators and samples, and most importantly, contains the glucoronide. The efficiency of hydrolysis for analyte and IS in samples is incomplete compared to calibrators, but the ratio is identical (2:1), thus the IS corrects for the efficiency difference observed in sample preparation, together with injection, separation and ionization.
Slide 10: Physicochemically identical AND Analytically identical..that’s a lot of deuterons that can scramble. Analyte should not contribute to IS and vice versa
Slide 11: Isotopes are a concern – some particularly likely candidates are Carbon and Sulphur. Analyte should not contribute to IS..when multiple Chlorines present..walk away to ensure contribution from analyte to the IS transition is non-existent BY DESIGN
Slide 12: Takes some consideration and even when planned perfectly..there can still be under-incorporation and/or loss..Analyte should not contribute to IS if at ALL possible
Slide 13: Analysis of small molecule panels such as amino acids is challenged with many analytes across a narrow mass range. While Methionine generates a major product ion at mass-to-charge 104, a contribution to the D3-Lysine transition of 150 to 87 is observed.. Making life even more complicated, The D5-Glutamic acid internal standard contributes to the D3-Methionine transition. Addition of D3-Proline with a transition from mass-to-charge 119 – 73 is not selectively measured by the mass spectrometer as the carbon 13 isotope of Valine also contributes to the transition. There are three solutions to this phenomenon. Select a different IS, or, add a lot of IS for proline and lysine to minimize the contribution (not ideal), or – resolve chromatographically – which is the correct solution.
Slide 14: Correctly selected IS defines analyte peak properties of retention time and shape
Slide 15: IS added after hydrolysis can correct for differences in injection volume, chromatography retention time variance and ionization differences – but – does not correct for the preparation efficiency difference between calibrators and samples, Bias still present.
Slide 16: QC level 1 injection 12 versus injection 94...D4 IS tells you – where analyte elutes and the shape of the eluted peak, so LC was working, IS response is identical between injection #12 and #94, so interface and mass spectrometer were working, but, the analyte transition shows elevated baseline NOT seen in the IS transition...shows contamination of LC-MS/MS system over time that is either carry-over from previous high level analyte sample (NOT the case) or another contaminant extracted from the specimen that shares the same transition and elutes later in the assay (YES). Solution, used a third washing solvent to clean off the column between each injection
Slide 17: There is always a sweet spot – if you think about it
Slide 18: Re-injection rules out preparation but imprecise preparation really makes the evaluation of drift impossible
Slide 19: Observation of IS peak area drift across a run using a liquid handler and the same 8-tips for IS addition. Reinjected the first part of the plate – same LOW IS response observed (LC-MS/MS system operating OK). Added a carrier to IS solution and pre-wetted the tip: aspirate/dispense to IS solution container 3 times prior to first aspiration to the 96-well plate
Slide 20: Speed isn’t what you are looking for – it’s control
Slide 21: And here’s how you test for it
Slide 22: Look at both the ratio over time and the loss of the IS – to have confidence in your experimental conclusions
Slide 23: Should be pretty obvious...Carbon and Nitrogen isotopes are preferred. The Carbon – Deuteron bond is more acidic than the Carbon-proton bond..and that does matter...see later
Slide 24: It’s the perfect correction tool and other technologies such as clinical autoanalyzers would love to have this capability
Slide 25: To correct for every step in the assay, the IS should be added immediately after mixing and pipetting calibrators, QC’s and samples. The internal standard needs to experience the same environment as the analyte in calibrators and samples, thus, the goal of the IS is to be in the same equilibrium state as the analyte, free and bound to sample constituents such as proteins.
Slide 26: Oh yes... a lot of weird stuff happens.... O-o
Slide 27: Neat solution of D4-Dopamine was injected using an APCI source and a very large response for Dopamine was observed (>highest desired calibrator). After checking for contamination, purchasing new materials (and checking the label carefully), the position of deuteration drew our attention. It is in a very “active” region of the molecule and APCI involves proton donation through gas phase collisions. Potential solutions include reducing the amount of IS added, but we need to have 10 – 25x LLOQ so that a reproducibly measured response is observed in all samples. In this instance, 20 fold dopamine to D4-Dopamine response was seen – we cannot logically add IS < assay LLOQ and expect success. When we switched to the ESI source, we noted no contribution of the IS to analyte transition due to a fundamentally different ionization mechanism (solvent removal versus gas phase ionization). Labelling in the wrong place – last on – first off.
Slide 28: Ionization mode provides multiple precursor ions in positive ion mode – contributing to response variance. You want low IS variance to spot outliers
Slide 29: Drift requires some experimentation, and cheaply made materials are not your friend
Slide 30: This isn’t the only time we have observed this, Deuterated IS materials are really the least favorable label you can use
Slide 31: Biology meets chemistry and creates a mass spectrometry nightmare..keep watching
Slide 32: Told you.....Oxym/Dhc could potentially have a +1 amu isotopic contribution to Cod-d3 therefore we choose the Cod-d6. However in doing so we also had to consider the m+2 contribution of Cod-d6 to Oxym-d6 which in fact has a selective transition and no contribution. Dhc-d3 is m-1 to Cod-d6 however we are both chromatographically separated and we have a selective transition. Hydrocodone-d6 is m+4 to Oxym but only m-2 from Oxym-d6 and we preferred the d3 as we choose Cod-d6. With the Hyc-d3 there’s only +1 amu difference to Oxym but through judicious transition selection we have a selective transition with no contribution of Oxym to Hyc-d3.
Slide 33: Historically, GC-MS assays tended to use heavily deuterated IS materials, in this case, Gabapentin IS contains 10 deuterons and the carbon – deuteron bond is more acidic than the carbon – proton bond. This can result in the IS eluting earlier than the analyte in reverse phase LC. Addition of equal amounts of Gabapentin and D10-Gabapentin to 150 urine samples demonstrates the issue with this. When they co-elute, the recovery ratio is generally 100% (1:1). When a slower LC separation is used, the IS does not co-elute with the analyte, resulting in many samples recovering much lower than expected. The answer isn’t fast LC – you lose selectivity, it’s a better labelled IS, ideally with carbon-13 or nitrogen-15 isotopes.
Slide 34: dynamic range (3-logs) the analyte contributes to the IS transition, so non-linear calibration curve seen. There are many solutions to this – but, do you want to run each sample with and without dilution, or repeat on dilution, or add more IS material to minimize contribution from analyte (you must have no unlabelled analyte in the IS for this), or find an alternate IS..Clinically the normal range is unaffected by the contribution in the linear part of the calibration range...so..ask your medical director. If you see this – LOOK at lower yield (3-5 fold here) transitions and see if you observe same degree of non-linearity in their curves:
IF Yes: Isotopic contribution, Preparative error, source saturation affecting IS response
IF NO: Detector blinding Using High QC (~80% ULOQ), assay neat and on dilution into “linear” portion – determine bias (<15% OK). Add calibrator(s) to define non linearity...OR...Solution - truncate linear range, pre-dilute samples, modify collision energy...or ask yourself if it is Clinically acceptable
Slide 35: Mass spectrometers are mixture analysis tools – BUT response (as transitions) is very different and further exacerbated when measuring mixtures with different circulating concentrations
Slide 36: De-convoluting the observation measuring multiple analytes in a single run really needs solid foundations in analytical chemistry to know what your eyes are telling you isn’t an issue...remember..Of the 5 senses, we trust our vision the most...but our eyes are the most easily fooled
Slide 37: As stated before..fast isn't good, good is good and fast comes as a side benefit
Slide 38: While many would argue this point..and it is my humble opinion...you will see later why imprecision should always rule
Slide 39: Fast isn't better...see..told you...
Slide 40: But fast can be achieved if you work the problem some..
Slide 41: Why does biology make life so...blurry...?
Slide 42: Because...depending upon what you believe..Biology has had a 5 billion or 5000 year head-start on scientists like us
Slide 43: But chemical understanding to ameliorate biology is a unique trait in humans...right?
Slide 44: Open your mind...legally of course
Slide 45: So what do you do about low IS? Have a policy...go conservative and re-extract? what if you can't? Really not result out for a patient that really needs help?
Slide 46: Hope you like the colors...I'm a fan of purple..and chose the colors as the obvious mixture of the two of each pair..yep..I take this that seriously
Slide 47: Its Expensive..but sooo worth it (I use that line with my wife quite a lot...sadly she does the same to me..)
Slide 48: Calibration frequency is a pain when STAT analysis is needed and doesn't have to be done by regulations as frequently as you would think...see The March of The Masses..Grant RP, Clin Chem, 2013 Jun;59(6):871-3. doi: 10.1373/clinchem.2013.205435. Epub 2013 Apr 16.
Slide 49: Simple to validate and use QC's to monitor – the stability and storage of the IS...as a component of the entire protocol
Slide 50: Same principal applies...its worth it just to see the look on the face of the auditors .. 😊
Slide 51: Hmm...what to do what to do...when samples are >ULOQ...really thaw, dilute, transfer, mix, cook for 2.5 hours etc...or...read next page?
Slide 52: We do this A LOT! Honestly
Slide 53: An exemplar..not technically accurate but relatable..pretty much like teaching the plum pudding model of the atom to kids...not that you are kids or anything..that's merely an analogy of the concept of an analogy..as a teaching tool...erm...moving on.....
Slide 54: nd this can be VERY VERY accurate..the foundation of what I would call proper reference method procedures..thanks to all of our colleagues who do this for us..truly great of you
Slide 55: Now this is quite cool if I say so myself...
Slide 56: No external calibrators, each samples recovery an matrix effects are “intrinsically” corrected...see what I did there...yep..its cool
Slide 57: And works like a charm
Slide 58: This took 3 years..while conceptually obvious..the metrology required to prove this was very painstaking..thanks to many groups who helped on this one...
Slide 59: Just look at the next slide and this one...accurate you say? When two peptides from the same protein give the same number from tryptic IS added as a “calibrator” they are CONCORDANT..not accurate..
Slide 60: Right..got off my soap box.....materials can be used as internal standards though..just don’t forget what you give up based on their form and where they are added...t make life more complicated...large protein IS materials are ANALOGS..because big proteins aren’t an entity, theyre a family of things..and we all know what secrets families hide..right?...erm....just mine then?
Slide 61: If you’re still reading...here’s a joke...

“As a kid I was made to walk the plank. We couldn’t afford a dog.”

Okay here’s one... “I was watching the London Marathon and saw one runner dressed as a chicken and another runner dressed as an egg. I thought: ‘Ooh, this could be interesting”.

Erm...“I’m sure wherever my dad is; he’s looking down on me. He’s not dead or tall, just very condescending."

😊 cheers, russ