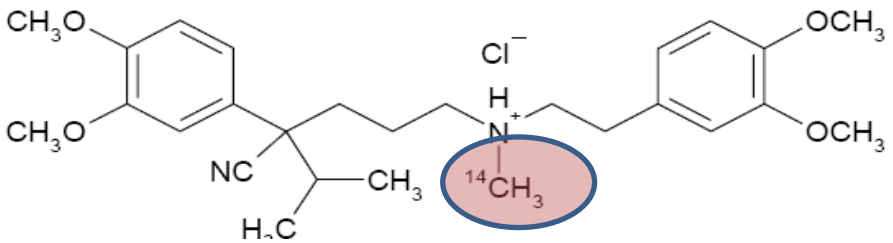
# What are the Critical Factors Determining the Performance of an LC+AMS Assay?

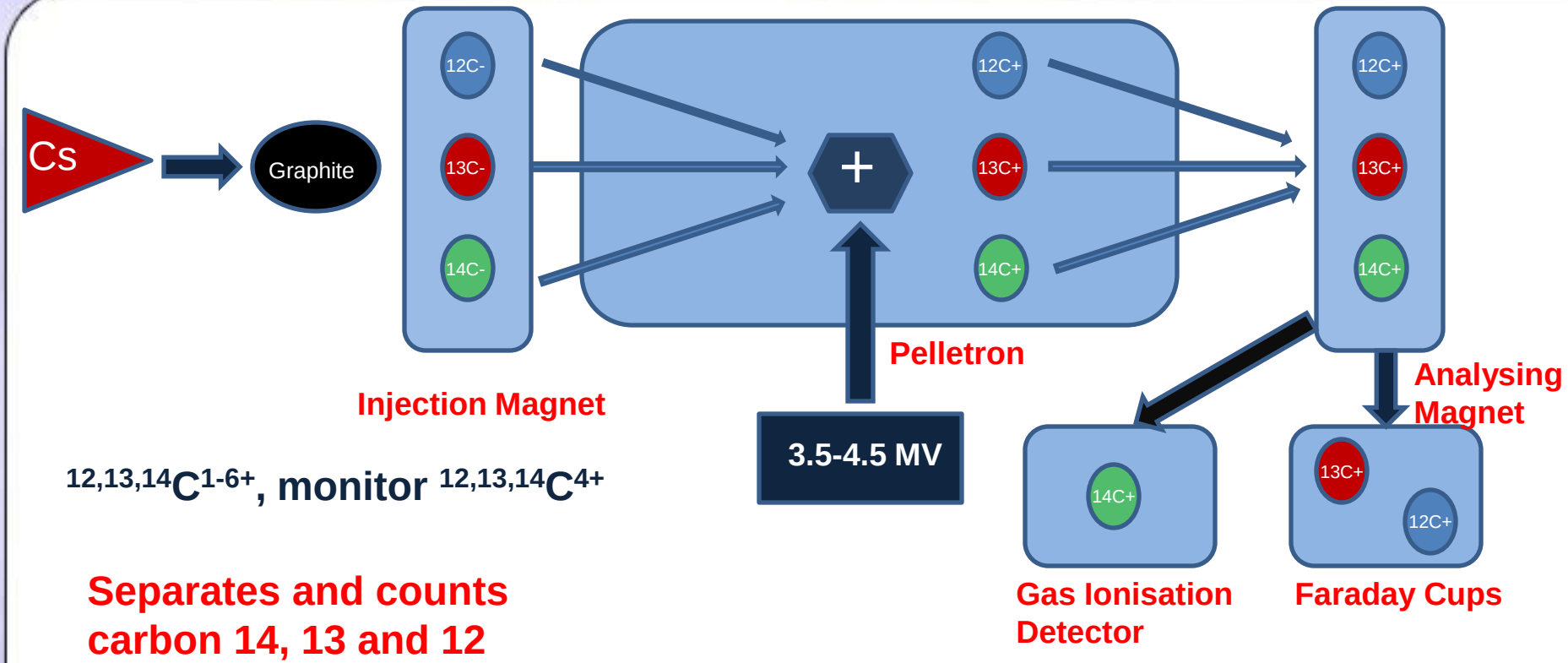
Dr Stuart Best, Senior Director of Operations



# Topics Covered in Presentation

- What is AMS?
- LC-MS and LC+AMS: the similarities and differences.
- LC+AMS Assay: the approach followed at Xceleron.
- Assay Validation of 14C-Verapamil
  - AMS specific considerations.
  - Validation parameters (response function, LLOQ, accuracy & precision, stability, matrix effects, selectivity and non-calibration line quantitation).
- Summary

# Accelerator Mass Spectrometer




$^{12,13,14}C^{1-6+}$, monitor $^{12,13,14}C^{4+}$

**Separates and counts carbon 14, 13 and 12 atoms.**
**Determines the ratio of $^{14}C$:$^{12}C$.**

**Carbon 14 has a very low natural abundance, $10^{-10}$ %.**

# LC-MS and LC+AMS: Similarities and Differences!

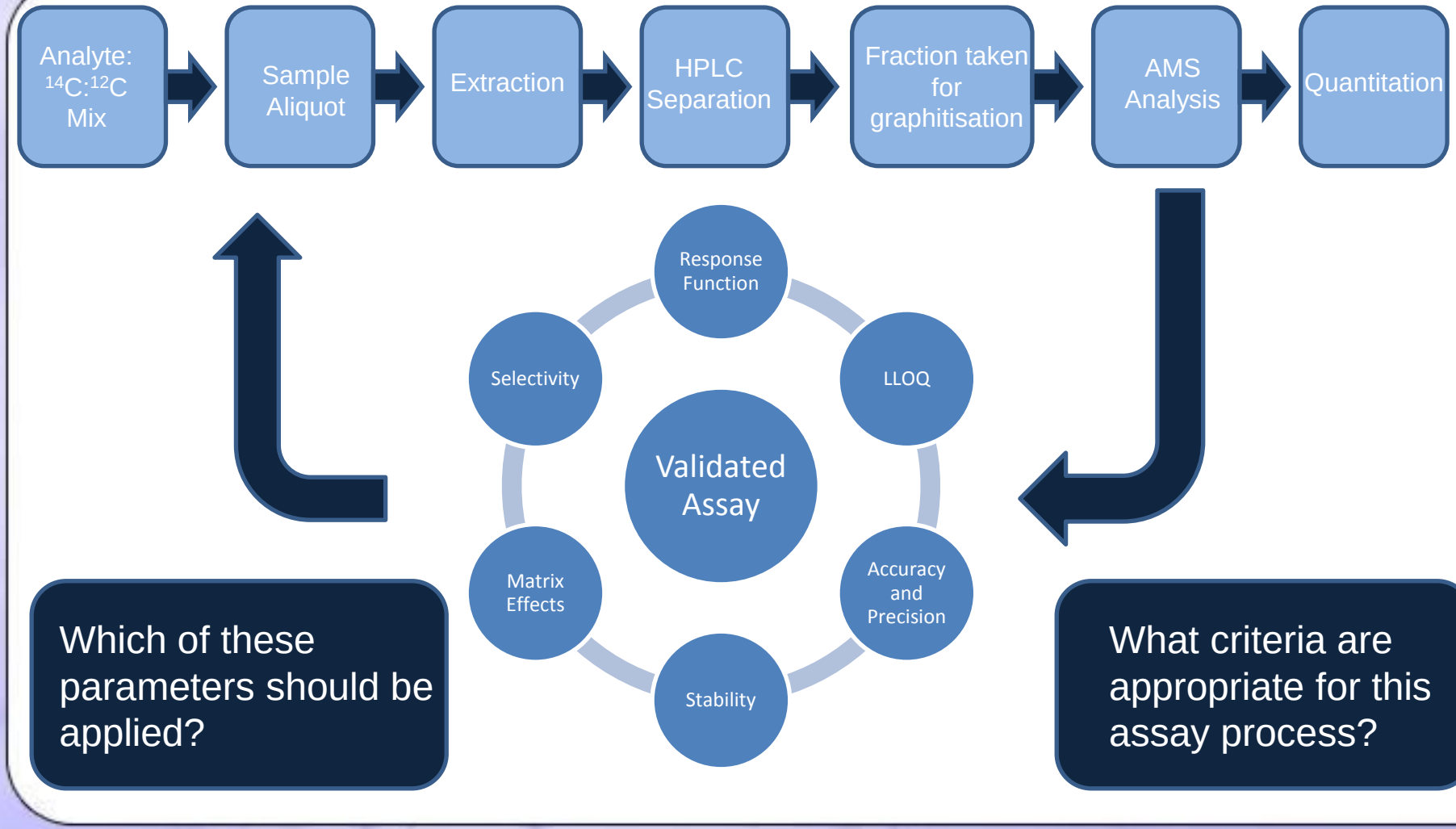
LC-MS

- Sensitivity (structural dependent).
- Matrix effects.
- Speed:
  - On-line.
- Structural Information:
  - selectivity/specificity. Monitor Q1 mass, Q3 mass and the transition between these two, in addition to a retention time.

LC+AMS

- High sensitivity (independent of structure).
- No matrix effects.
- Speed:
  - Off-line: HPLC fractionation => graphitisation = > AMS analysis.
- Structural Information lost:
  - Selectivity a combination of the presence of $^{14}C$ and chromatographic resolution.

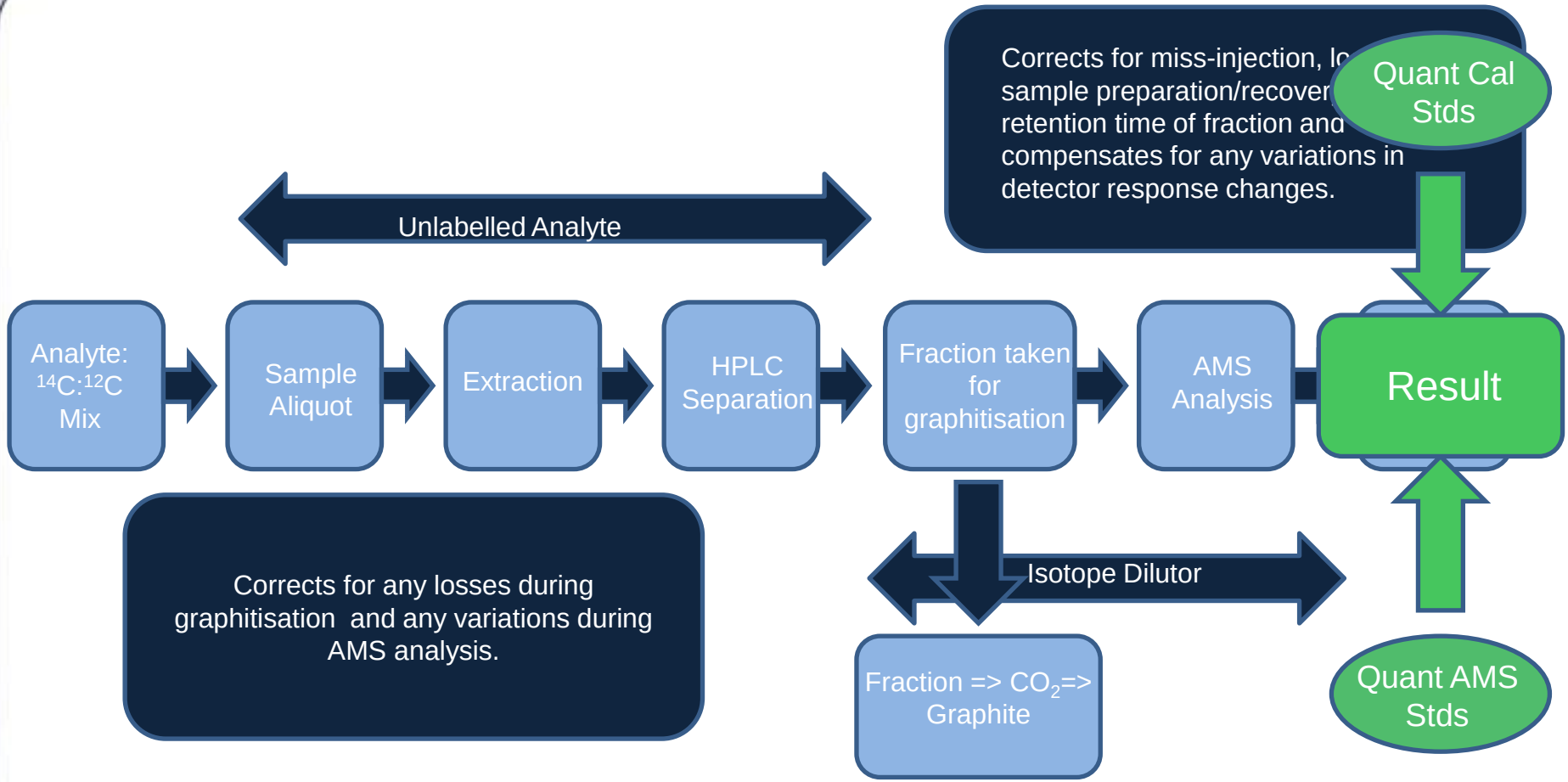
# The LC+AMS Assay



# Specific Considerations for an AMS Assay

- $^{14}C$: ubiquitous in our environment, assay operating below background amounts.
  - Careful controls in laboratory to segregate highly radioactive samples.
  - Process and procedures to minimise cross contamination (from environment).
- Homogeneity of Validation samples.
  - Analyte is a mixture of $^{14}C$-analyte plus $^{12}C$, often with the $^{12}C$ analyte in considerable excess.
  - When spiked into plasma, how do you achieve equilibrium of the two entities?
  - $^{14}C$ is present at very low amounts, need to consider the potential of non-specific binding (eg add internal standard in excess).

# AMS Assay Process



# Response Function

- The dynamic range of the AMS is known from detection above background to the amount that will saturate the detector (2 dpm). This is independent from chemical structure.
- The AMS determines a $^{14}C$:$^{12}C$ ratio (pMC) and the HPLC detector (usually uv) determines peak area.
- A composite response function is created of pMC/peak area.
- This ratio can be plotted against nominal concentration to form a calibration line.
- Quantitation can also employ measurement with reference to a certified standard (AMS calibration) corrected for recovery (recovery constant).

# Response Function: Quantitation

- Quantitation employing a calibration line.
- AMS data can be heteroscedastic (SD of data is proportional to the magnitude of the response being measured) and data should be tested to determine if weighted linear regression is appropriate .
- Acceptance criteria applied for accuracy are ±25%* (LLOQ), ±20%*, with 75% of standards analysed meeting this criterion. These are wider criteria than standard guidance but reflect the off-line nature of this approach.
- Lines are composed of 6-8 standards, single or duplicate.
- If data are determined to be heteroscedastic, a weighting of 1/X is applied.

# Response Function of $^{14}$C-Verapamil

| Nominal Matrix Concentration (dpm/mL) | Calculated Matrix Concentration (dpm/mL) | | | | Accuracy (%) | Precision (%) |
|---|---|---|---|---|---|---|
| | Batch 1 | Batch 2 | Batch 3 | Mean | | |
| 0.04758 | 0.0421 | 0.0490 | 0.0452 | 0.0454 | **95.4** | **7.6** |
| 0.09516 | 0.0867 | 0.0763 | 0.0925 | 0.0852 | **89.5** | **9.6** |
| 0.1903 | 0.214 | 0.193 | 0.185 | 0.197 | **103.5** | **7.6** |
| 0.4758 | 0.582 | 0.490 | 0.528 | 0.533 | **112.0** | **8.7** |
| 0.9516 | 0.976 | 1.01 | 1.02 | 1.00 | **105.1** | **2.3** |
| 1.903 | NR | 1.94 | 1.72 | 1.83 | **96.2** | **8.5** |
| 4.758 | 5.31 | 5.32 | 4.81 | 5.14 | **108.0** | **5.7** |
| 9.516 | 8.93 | 8.87 | 9.53 | 9.11 | **95.7** | **4.0** |
| Slope | 0.074141 | 0.0702660 | 0.0886340 | 0.0776803 | | **12.5** |
| Intercept | 0.001185 | 0.001607 | 0.001225 | 0.001339 | | **17.4** |

NR=No result

**$^{14}$C-analyte 0.172-34.3 pg/mL (total mass *ca* 4-900 pg/mL)**

# Lower Limit of Quantitation (LLOQ)

- The lowest concentration at which acceptable accuracy and precision is displayed: Mean accuracy of ±25%, with a precision of <25%, n=6 replicates.
- Need to also consider the amount of background $^{14}C$ of the process (determined from the isotope dilutor), background of blank control plasma and ensure subjects are free from excessive $^{14}C$ (from other radiolabelled studies!).
- Also need to consider the separation of the background signal from that determined at the LLOQ, set at a 5 fold window (target).

# Accuracy and Precision

- Determined at 2-3xLLOQ, mid-range, and 75% of top standard, n=6 replicates and on three occasions.
- Acceptance criteria are mean accuracy of ±20% for intraday and interday determinations, with precision of <20% for both intra and interday measurements.
- Failed Samples: samples can also be rejected based on low current (insufficient graphite), because they are too radioactive (system terminates measurement) or due to an abnormal $^{13}C$:$^{12}C$ ratio . These parameters are monitored for all samples during validation and routine sample analysis to monitor correct performance of the AMS.

# Accuracy and Precision of $^{14}$C-Verapamil Assay

| | Replicate | Batch 1 | | | | Batch 2 | | | | Batch 3 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Nominal Concentration (dpm/mL) | | | | Nominal Concentration (dpm/mL) | | | | Nominal Concentration (dpm/mL) | | | |
| | | 0.04758 | 0.09515 | 0.7135 | 7.135 | 0.04758 | 0.09515 | 0.7135 | 7.135 | 0.04758 | 0.09515 | 0.7135 | 7.135 |
| | | Calculated Concentration (dpm/mL) | | | | Calculated Concentration (dpm/mL) | | | | Calculated Concentration (dpm/mL) | | | |
| | 1 | 0.0499 | 0.101 | 0.816 | 7.79 | 0.0543 | 0.0798 | 0.822 | 8.03 | 0.0342 | 0.0833 | 0.781 | 7.33 |
| | 2 | 0.0517 | 0.094 | 0.768 | 7.27 | 0.0564 | 0.0992 | 0.872 | 8.16 | 0.0515 | 0.0860 | 0.806 | 7.68 |
| | 3 | 0.0543 | 0.106 | 0.871 | 7.81 | 0.0464 | 0.0954 | 0.805 | 7.78 | 0.0404 | 0.1050 | 0.778 | 7.51 |
| | 4 | 0.0514 | 0.093 | 0.845 | 7.69 | 0.0454 | 0.0861 | 0.870 | 7.71 | 0.0465 | 0.0906 | 0.743 | 7.23 |
| | 5 | 0.0432 | NR | 0.815 | 7.49 | 0.0474 | 0.0834 | 0.838 | 7.63 | 0.0403 | 0.0813 | 0.821 | 6.61 |
| | 6 | 0.0497 | 0.100 | 0.846 | 7.46 | 0.0648 | 0.0759 | 0.821 | 8.10 | 0.0453 | 0.0930 | 0.788 | 7.42 |
| | Mean | 0.0500 | 0.09870 | 0.827 | 7.59 | 0.0525 | 0.0866 | 0.838 | 7.90 | 0.0430 | 0.0898 | 0.786 | 7.30 |
| | Accuracy (%) | **105.1** | **103.7** | **115.9** | **106.4** | **110.3** | **91.0** | **117.4** | **110.7** | **90.4** | **94.4** | **110.2** | **102.3** |
| | Precision (%) | **7.5** | **5.4** | **4.3** | **2.8** | **14.3** | **10.4** | **3.3** | **2.8** | **14.0** | **9.6** | **3.4** | **5.1** |
| Interday | Mean | 0.0485 | 0.09135 | 0.81700 | 7.59 | | | | | | | | |
| Interday | Accuracy (%) | **101.9** | **96.0** | **114.5** | **106.4** | | | | | | | | |
| Interday | Precision (%) | **14.3** | **9.9** | **4.5** | **4.8** | | | | | | | | |

NR=No result

**LLOQ: 0.172 pg/mL (4.44 pg/mL)**
**Low: 0.344 pg/mL (8.88 pg/mL)**
**Medium: 2.58 pg/mL (66.58 pg/mL)**
**High: 25.78 pg/mL (665.8 pg/mL)**

# Stability Experiments

- Performed with n=3 replicates at low and high concentrations, with acceptance criteria of mean accuracy of ±20% of nominal.
- Matrix stability, autosampler stability and freeze-thaw stability.

| Replicate | Concentration (dpm/mL) | | | | | |
|---|---|---|---|---|---|---|
| | 0.09515 | | | 7.135 | | |
| | | 4°C | Room Temp | | 4°C | Room Temp |
| | 0 | 22h | 22h | 0 | 22h | 22h |
| 1 | 0.107 | 0.0716 | 0.0801 | 7.41 | 6.24 | 6.45 |
| 2 | 0.104 | 0.0960 | 0.0737 | 8.16 | 6.81 | 6.94 |
| 3 | 0.0963 | 0.0875 | 0.0796 | 8.08 | 6.95 | 6.87 |
| Mean | 0.102 | 0.085 | 0.0778 | 7.89 | 6.67 | 6.75 |
| Accuracy (%) | **106.0** | **88.3** | **80.8** | **97.6** | **82.5** | **83.5** |
| Precision (%) | **5.4** | **14.6** | **4.6** | **5.2** | **5.6** | **3.9** |

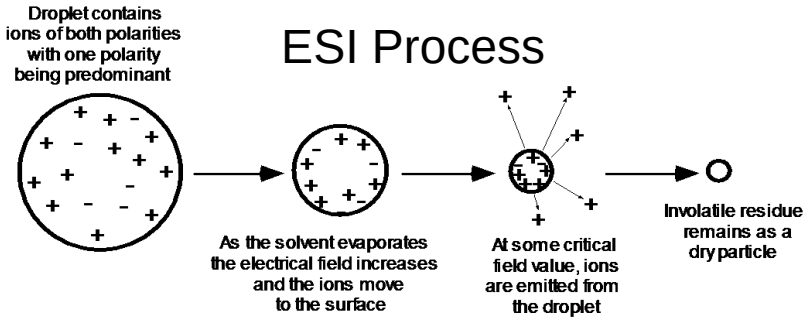
| Replicate | Concentration (dpm/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0.09515 | | | | 7.135 | | | |
| | Cycle | | | | Cycle | | | |
| | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 |
| 1 | 0.107 | 0.0798 | 0.184 | 0.0825 | 7.41 | 8.03 | 6.89 | 8.18 |
| 2 | 0.104 | 0.0992 | 0.108 | 0.0965 | 8.16 | 8.16 | 7.67 | 8.04 |
| 3 | 0.0963 | 0.0954 | 0.115 | 0.0894 | 8.08 | 7.78 | 7.53 | 8.92 |
| Mean | 0.102 | 0.0915 | 0.112 | 0.0895 | 7.89 | 7.99 | 7.36 | 8.38 |
| Accuracy (%) | **107.2** | **96.2** | **117.7** | **94.1** | **110.6** | **112.0** | **103.2** | **117.4** |
| Precision (%) | **5.4** | **11.2** | **4.4** | **7.8** | **5.2** | **2.4** | **5.7** | **5.6** |

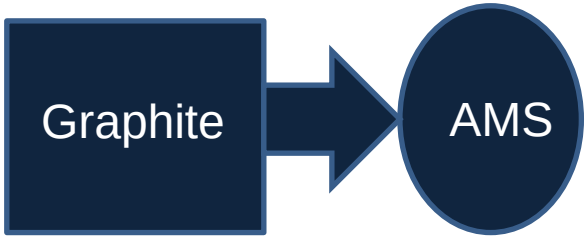
Not used

- Require by AMS detection or inferred from LC-MS Assays (EMA Guidance).

# Matrix Effects

- Samples are extracted (separation), fractionated (separation), combusted and cryogenically transferred (separation) and then reduced to graphite.




- Lack of matrix effects permits simple sample preparation approaches such as protein precipitation without the need for elaborate clean-up strategies and testing for matrix effects or interferences for co-administered medicines.

# Selectivity

- Dependent upon the presence of $^{14}C$ and the ability of the chromatographic separation to resolve $^{14}C$ analyte from metabolites/ degradants.
  - Confirmed by analysis of reference standards of metabolites.
  - Probed by alternative chromatographic systems.
  - Collect narrow fractions at 15 secs and examine peak shape.
  - Confirm peak purity by LC-MS?
- As matrix effects are very unlikely, multiple sources of plasma are not required.

# Quantitation with Recovery Constant (Mean)

| | Replicate | Batch 1 | | | | Batch 2 | | | | Batch 3 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Nominal Concentration (dpm/mL) | | | | Nominal Concentration (dpm/mL) | | | | Nominal Concentration (dpm/mL) | | | |
| | | 0.04758 | 0.09515 | 0.7135 | 7.135 | 0.04758 | 0.09515 | 0.7135 | 7.135 | 0.04758 | 0.09515 | 0.7135 | 7.135 |
| | | Calculated Concentration (dpm/mL) | | | | Calculated Concentration (dpm/mL) | | | | Calculated Concentration (dpm/mL) | | | |
| | 1 | 0.04732 | 0.097166 | 0.786963 | 7.534 | 0.0549387 | 0.080501 | 0.82798 | 8.115 | 0.03520 | 0.083979 | 0.774168 | 7.261 |
| | 2 | 0.04864 | 0.091341 | 0.741896 | 7.031 | 0.0520349 | 0.101268 | 0.876522 | 8.249 | 0.05084 | 0.087003 | 0.79932 | 7.610 |
| | 3 | 0.05150 | 0.102488 | 0.8415 | 7.548 | 0.0443577 | 0.09289 | 0.8096 | 7.857 | 0.04221 | 0.105563 | 0.770846 | 7.435 |
| | 4 | 0.04898 | 0.090487 | 0.815529 | 7.434 | 0.0442744 | 0.085759 | 0.878153 | 7.863 | 0.04782 | 0.090932 | 0.737075 | 7.164 |
| | 5 | 0.04246 | NR | 0.788051 | 7.238 | 0.0467565 | 0.084971 | 0.846241 | 7.710 | 0.04171 | 0.082298 | 0.815079 | 6.540 |
| | 6 | 0.04814 | 0.095972 | 0.817479 | 7.216 | 0.0648193 | 0.07555 | 0.828659 | 8.186 | 0.04635 | 0.094073 | 0.782074 | 7.339 |
| | Mean | 0.04784 | 0.09549 | 0.79857 | 7.33323 | 0.05120 | 0.08682 | 0.84453 | 7.99661 | 0.04402 | 0.09064 | 0.77976 | 7.22484 |
| | Accuracy (%) | **100.5** | **100.4** | **111.9** | **102.8** | **107.6** | **91.2** | **118.4** | **112.1** | **92.5** | **95.3** | **109.3** | **101.3** |
| | Precision (%) | **6.2** | **5.1** | **4.3** | **2.8** | **15.5** | **10.5** | **3.3** | **2.7** | **12.6** | **9.4** | **3.4** | **5.1** |
| Interday | Mean | 0.04769 | 0.09072 | 0.80762 | 7.51823 | | | | | | | | |
| Interday | Accuracy (%) | **100.2** | **95.3** | **113.2** | **105.4** | | | | | | | | |
| Interday | Precision (%) | **13.1** | **9.0** | **4.9** | **5.8** | | | | | | | | |

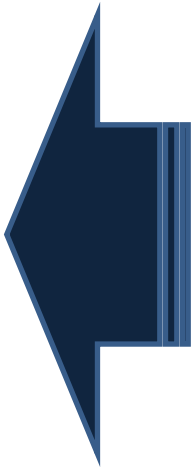
NR=No result

**LLOQ: 0.172 pg/mL (4.44 pg/mL)**
**Low: 0.344 pg/mL (8.88 pg/mL)**
**Medium: 2.58 pg/mL (66.58 pg/mL)**
**High: 25.78 pg/mL (665.8 pg/mL)**

**Quantitation based on an assay independent reference standard of $^{14}C$ material (AMS Calibration).**
**Recovery Constant=**
**(Fraction Recovered/Nominal Conc)/Peak Area**

# Batch Variation of Recovery Constant

| Nominal Conc dpm/mL | Recovery Constant | | | Mean | CV (%) |
|---|---|---|---|---|---|
| | Batch 1 | Batch 2 | Batch 3 | | |
| 0.04758 | 0.00031103 | 0.00032301 | 0.00040630 | 0.00034678 | **15.0** |
| 9.516 | 0.00032094 | 0.00030189 | 0.00040927 | 0.00034404 | **16.7** |
| 4.758 | 0.00038111 | 0.00036164 | 0.00041328 | 0.00038534 | **6.8** |
| 1.903 | No result | 0.00032954 | 0.00037023 | 0.00034989 | **8.2** |
| 0.9516 | 0.00035066 | 0.00034161 | 0.00044083 | 0.00037770 | **14.5** |
| 0.4758 | 0.00041600 | 0.00033134 | 0.00045360 | 0.00040031 | **15.6** |
| 0.1903 | 0.00038419 | 0.00032706 | 0.00040293 | 0.00037140 | **10.6** |
| 0.09516 | 0.00031082 | 0.00024809 | 0.00040555 | 0.00032149 | **24.7** |
| Mean | 0.00035354 | 0.00032052 | 0.00041275 | | |
| CV (%) | **11.7** | **10.5** | **6.1** | | |



- Run a recovery constant with each batch samples to compensate for variation.

# Summary

- This LC+AMS assay can be validated against the standard bioanalytical criteria, as demonstrated for $^{14}$C-verapamil.
- It is an off-line techniques with sample manipulation during graphitisation (a sample preparation step) and thus wider accuracy and precision are appropriate (accuracy ±20%, precision, <25%).
- Containment processes are critical:
  - $^{14}$C in the environment, monitor system, reagents, plasma.
  - Specific laboratory practices to minmise carryover/cross contamination.
  - Define background of process and separation between this and LLOQ.

# Summary

- AMS has a known linear range independent from structure and does not suffer from matrix effects to the degree LC-MS does.
- Quantitation can be achieved with either a calibration line or recovery constant approach/AMS calibration. Weighted linear regression is appropriate for this example.
- The testing for matrix effects is not required.
- Can stability be inferred from LC-MS data at similar concentrations (EMA Guidance)?
- HPLC resolution is critical, to ensure assay specificity.