Quantitative work in HPLC

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Quantitative work in HPLC

LAYOUT

• Standards Considerations
• Integration events: Explained and demonstrated
• Running sets of samples
  – Blanks – Carry over and system peaks
  – System Suitability
  – Calibration and Quantitation
• Parameters effecting quantitation and validation
• Requirements from chromatographic data systems
Quantitative work in HPLC

- Standards Considerations
  - Integration events: Explained and demonstrated
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REFERENCE STANDARDS:

2.0 DESCRIPTION OF CRM
   Custom-Grade 10000 µg/mL Lead in 0.35% (abs) HNO₃
   Catalog Number: CGP810-1 and CGP810-5
   Lot Number: T-PB02113
   Starting Material: Pb(NO₃)₂
   Starting Material Purity (%): 99.9997
   Starting Material Lot No: 22160
   Matrix: 0.35% (abs) HNO₃

3.0 CERTIFIED VALUES AND UNCERTAINTIES
   Certified Concentration: 10.099 ± 2 µg/mL
   Certified Density: 1.014 g/mL (measured at 22°C)
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Standard Information

4.7.1 The working standard bottles contain the following details:

⇒ Name
  • Stock no.
  • Catalog no.
⇒ Expiry date
⇒ Purity % (as is)
⇒ Water %
⇒ Storage conditions (if special conditions are required)
⇒ Special care before use (e.g. drying, water determination)

* If the material is hygroscopic, extra precautions are required when drying the sample prior to use.

Standards in the Industry

Reference Standards

Primary Standards

Pharmacopoeial standards
Source: Pharmacopoeia Commission

Primary Standards
Source: Suppliers

Working Standards

Impurity Standards
Primary Standard

Materials which are accepted without reference to other standards. If the materials have undergone complete analytical characterization, their identity must be proven (elucidation of chemical structure) and their purity must be sufficiently high and stated (>99.0%).

The characterization of primary standards generally involves the following:

- Elucidation of chemical structure by IR, UV, H-NMR, C-NMR, MS, CD etc.
- Purity determination by HPLC, TLC, GC, GPC, DSC, residue of ignition, water content etc.
- Assay: Titration, DSC, Chromatography.

It is acceptable that the manufacturing process of primary references standards differs from the final processing of the drug substance.

Working Standard

Materials are designed for daily use in instrumental analysis such as routine quality control.

They are characterized by comparison with Primary or Pharmacopoeia standards. Their purity corresponds to a "typical batch".

Pharmacopeial Standards
Commonly used for certain tests and assayed to achieve accuracy and precision of analytical results required in compendia monographs. Pharmacopeial standards are basically regarded as primary standards.
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Information Supporting The Integrity of The Reference Standard

- Tests to characterize the reference standard are different and more extensive than those used to control the new drug substance as to Identity, Strength, Quality, and Purity
- Physical description
- Physical constants (RI, pK, BP, MP, etc.)
- Chemical Attributes (Formula, weight)
- Appropriate analytical tests used to ID
- Data establishing purity

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Integration

Measurement of Peak Area - Integration

\[
\text{Area} = \int_{\text{Start}}^{\text{End}} \text{Abs} \times dt
\]
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Measurement of Area:
Peak Integration

Data Bunching
Peak Start
Peak Apex
Peak End

Peak Detection – Integration Events

Start of chromatogram
Peak Start
Retention Time
Peak Apex
Peak Height
Constructed Baseline
Peak End
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**Influence of Poor Peak Shape on Integration (Peak Area)**

- Tailing Factor = 1.00
- Recovered Peak Areas:
  - 99.9%
  - 99.8%
  - 99.6%

- Tailing Factor = 1.58
- Recovered Peak Areas:
  - 97.8%
  - 95.3%
  - 92.3%

**Measurement of Area: Peak Integration: Data Bunching**

15 = Minimum Number of points to define a peak

Bunching

\[ B = \frac{W \times S}{15} \]

- \( B \) = BUNCHING FACTOR
- \( W \) = PEAK WIDTH
- \( S \) = SAMPLING RATE
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Measurement of Area:
Peak Integration - Peak Start

Slope 1 = \((B2-B1)/(t2-t1)\)
Slope 2 = \((B3-B2)/(t3-t2)\)
Average Slope

B1 → B3
Threshold value

Measurement of Area:
Peak Integration - Peak End

Slope 1 = \((B2-B1)/(t2-t1)\)
Slope 2 = \((B3-B2)/(t3-t2)\)
Average Slope

B1 → B3
Threshold value
(Touchdown)
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Measurement of Area:

Peak Integration - Peak Apex

\[ \text{Slope 1} = \frac{(B2-B1)}{(t2-t1)} \]

\[ \text{Slope 2} = \frac{(B3-B2)}{(t3-t2)} \]

Proper Integration Events are Required

![Graph showing measurement of area in HPLC](image-url)
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Set Peak Width

Peak Width = 30

Peak Width = 120

Peak Threshold

Peak Threshold = 300

Peak Threshold = 75
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When a riding peak needs to be skimmed from the slope there are two options, exponential or tangential skim:

Exponential Skim

Tangential Skim

Force Baseline by Time
& Force Baseline by Peak

A gradient chromatogram, where the baseline is drifting:

Event start and end times (Force Baseline by Time)
Peak start and end points within this range (Force Baseline by Peak)
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Force Drop line

Originally

Force Drop line

Valey to Valey

Original

Valey to Valey
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Asymmetric factor or Tailing factor

Sources of Errors in Integration of Asymmetric Peaks

Slope 1 = \((B_2 - B_1)/(t_2 - t_1)\)

Slope 2 = \((B_3 - B_2)/(t_3 - t_2)\)

Average Slope = \(\frac{Slope_1 + Slope_2}{2}\)

Threshold value
Integration of Small Peaks

- AUFS = 0.003
- Peak width changed
- Threshold set at 30

- AUFS = 0.003
- Peak width set at 30 sec
- Threshold changed

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- **Standards Considerations**
- **Integration events:** Explained and demonstrated

  - Running sets of samples
    - Blanks – Carry over and system peaks
    - System Suitability
    - Calibration and Quantitation

- Parameters effecting quantitation and validation
- Requirements from chromatographic data systems
Running Sets of Samples in Regulated Environment

Basic Sequence in HPLC

1. Login.
2. Instrument Method.
3. Sequence/Run Samples.
5. Review/Preview.
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FDA Guidance

- Table 2. Holistic Validation of Computerized LC Systems
  - Initial Calibration--Linearity
    - Use at least 4 standard solutions.
    - Concentration range of standards must span anticipated results, plus safety margin.
    - Run standards daily before starting sample analysis.
  - Initial Calibration--System Precision
    - Calculate precision daily from at least 6 replicate injections of a standard solution before starting sample analysis.
  - Running Calibration
    - Run a standard solution at specified time intervals or after a specified number of sample solutions.
  - Data Processor (Integrator, Computer)
    - Are the macros coded correctly? Validated? Documented?
  - System Documentation
    - Is System Documentation in Place?

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### Sample vs. Blank (Diluent)

Unexpected peaks can appear in blanks and in all following injections. In this case they are excluded from the processing of the sample.
INJECTION OF PURE SOLVENT:
Why would there be peaks?

System Peaks

“Legitimate” System Peaks
Originate from the Mobile Phase Components Going Through Re-Equilibration
CONDITIONS FOR APPEARANCE OF REAL SYSTEM PEAKS

- Mobile phase is multi-component ($n \geq 2$)
- Mobile phase contains adsorbable components
- Mobile phase's components respond to the detector (high background)
- Sample or sample-diluent is different from the mobile phase, enough to create equilibrium perturbation.

**Mechanism of System Peaks Formation**

**Example:** Two additives in the mobile phase

- **Example: $k'(1) = 1$**
  - **Step 1:**
    - Equilibrium:
      - $C_s = 1$; $C_m = 1$
  - **Step 2:**
    - Injection of Vacancy:
      - $C_s = 1$; $C_m = 0$
  - **Step 3:**
    - Re-equilibration:
      - $C_s = 0.5$; $C_m = 0.5$

- **Example: $k'(2) = 2$**
  - **Step 1:**
    - Equilibrium:
      - $C_s = 2$; $C_m = 1$
  - **Step 2:**
    - Injection of Vacancy:
      - $C_s = 2$; $C_m = 0$
  - **Step 3:**
    - Re-equilibration:
      - $C_s = 1.33$; $C_m = 0.67$

**CHROMATOGRAM**

\[
k' = \frac{t_R - t_0}{t_0}
\]

\[
k' = \phi \frac{C_s}{C_m}
\]
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An Example for Real System Peaks in Gradients with Trifluoroacetic Acid (TFA) in the Mobile Phase

System peaks appear because diluent does not contain TFA

Contaminations Peaks - Not System Peaks!
Phosphate Buffer and Acetonitril Do Not Produce Such System Peaks!

Mobile phase: ACN : Phosphate Buffer pH 9.5 1:1
Wavelength: 280 nm

Sample Name: Diluent

Minutes
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Use of Diode-Array Detector for Troubleshooting of Contamination Peaks in Multicomponent Mobile Phase

Chromatogram and Peaks' UV-VIS Spectra

Wavelength: 254.0 nm

Suggested Flow-Chart for Troubleshooting

In a Regulated Environment (no change in method!)

Injection → "System Peak" ???

Carry over ???

Contamination ???

Yes?

No

Mobile phase:
Multicomponent?
Adsorbed components (ion pair)?
Response in detector (background)?

Yes?

Legitimate System Peaks
• Dissolve samples in mobile phase
• Adjust diluent with mobile phase components
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Suggested Flow-Chart for Troubleshooting Contd.

Steps: by ease of use
1. Review and revise diluent’s composition
2. Replace vials’ batch and/or brand
3. Replace in-line filters
4. Replace solvents batch and/or brand
5. Try a new/fresh column
6. Wash system, especially injector
Make sure to try a fresh blank each test!

Not System Peaks?

Not washed by blanks?

Peaks still persist?
Replace injector’s surfaces

Peaks still persist?
Try another instrument/Operator/Lab

Many times the extraneous peak disappears on its own and remains an unsolved mystery…
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<table>
<thead>
<tr>
<th>VIAL</th>
<th>SAMPLE NAME</th>
<th>INJ VOL</th>
<th>No of Inj</th>
<th>Function</th>
<th>Method</th>
<th>Run Time</th>
<th>Sample Weight</th>
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<td>1.00000</td>
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<td>1.00000</td>
<td>1.00000</td>
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<td>1.00000</td>
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<td>Unk.6</td>
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<td>2</td>
<td>Inject Samples</td>
<td>LC Demo Method Set</td>
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<td>1</td>
<td>Inject Standards</td>
<td>LC Demo Method Set</td>
<td>10.00</td>
<td>1.00000</td>
<td>1.00000</td>
</tr>
</tbody>
</table>

Clear Calibration
Calibrate
## System Suitability

**Sample Name:** AntiOX 10.2d  
**Sample Type:** Unknown  
**Vial:** 2-A  
**Injection #:** 1  
**Injection Volume:** 2.00 μl  
**Run Time:** 2.0 Minutes  
**Date Acquired:** 19/10/2009 16:15:30 IST  
**Date Processed:** 14/12/2009 22:39:44 IST

### Auto-Scaled Chromatogram

**BHT:** 0.193  
**ENICAMIDE:** 0.303  
**IRGANOX 3114:** 0.459  
**IRGANOX 1010:** 0.552  
**VIT E:** 0.731  
**IRGANOX 1076:** 0.966  
**IRGAFOS 168:** 1.299

### Peak Results

<table>
<thead>
<tr>
<th>Name</th>
<th>RT</th>
<th>Area</th>
<th>Height</th>
<th>Plate Count</th>
<th>USP Plate Count</th>
<th>Tailing Alfa</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHT</td>
<td>0.193</td>
<td>97192</td>
<td>196749</td>
<td>1028</td>
<td>948</td>
<td>1.46</td>
</tr>
<tr>
<td>ENICAMIDE</td>
<td>0.303</td>
<td>88614</td>
<td>74300</td>
<td>1379</td>
<td>1419</td>
<td>3.84</td>
</tr>
<tr>
<td>IRGANOX3114</td>
<td>0.459</td>
<td>59739</td>
<td>131707</td>
<td>43447</td>
<td>116748</td>
<td>1.34</td>
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<tr>
<td>IRGANOX1010</td>
<td>0.552</td>
<td>77254</td>
<td>44992</td>
<td>2229</td>
<td>2287</td>
<td>6.37</td>
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<tr>
<td>VIT E</td>
<td>0.731</td>
<td>59739</td>
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<td>0.966</td>
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<tr>
<td>IRGAFOS 168</td>
<td>1.299</td>
<td>43447</td>
<td>18010</td>
<td>6540</td>
<td>6551</td>
<td>5.64</td>
</tr>
</tbody>
</table>

**Auto-Scaled Chromatogram**

**PEAK BROADENING**
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System Suitability

is

Based on the Theory of Chromatography

The Chromatographic Process

Elution through the Column

Distribution:

\[ K = \frac{C_s}{C_m} \]

Chromatogram

Area A = 300,000
Area B = 100,000

Abs

Time

Minutes

A - 93.448
B - 94.776
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Mixed-Mode Retention:

Hydrophobic Interaction with Bonded Phase

Ion exchange Interaction with Charged Sites

Mobile Phase pH < 3
Si - OH

Base

Mobile Phase pH > 3
Si – O –

Base

Influence of Poor Peak Shape on Integration (Peak Area)

Tailing Factor = 1.00
Recovered Peak Areas

\[ \text{\textbullet} \quad 99.9 \% \]
\[ \text{\triangle} \quad 99.8 \% \]
\[ \text{\triangle} \quad 99.6 \% \]

Tailing Factor = 1.58
Recovered Peak Areas

\[ \text{\textbullet} \quad 97.8 \% \]
\[ \text{\triangle} \quad 95.3 \% \]
\[ \text{\triangle} \quad 92.3 \% \]
System Suitability

- System Suitability
  - The checking of a system, before or during analysis of unknowns, to insure system performance.
  - “No sample analysis is acceptable unless the requirements for system suitability have been met.” (USP Chapter 621)
- Plate Count, Tailing, Resolution
- Determination of reproducibility (%RSD)
  - For %RSD < 2.0%, Five replicates
  - For %RSD > 2.0%, Six replicates
- System Suitability “Sample”
  - A mixture of main components and expected by-products utilized to determine system suitability
  - “Whenever There is a Significant change in Equipment or Reagents, System Suitability Testing Should be Performed” (USP Chapter 621)
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Standard Deviations and Areas Under the Normal Curve

- For any normal curve with mean \( \mu \) and standard deviation \( \sigma \):
  - 68 percent of the observations fall within ±1 standard deviation of the mean.
  - 95 percent of observations fall within ±2 standard deviations.
  - 99.7 percent of observations fall within ±3 standard deviations of the mean.

Measuring Peak Widths

\[
w = 2.355 \cdot \sigma \\
w = 4 \cdot \sigma \\
w = 5 \cdot \sigma
\]
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Efficiency method Vs. Peak width

TABLE 2.1 Factor $f$ of Equation (2.6) as Function of How Peak Width $w_p$ Is Measured

<table>
<thead>
<tr>
<th>Method</th>
<th>Position of Peak Width</th>
<th>Factor $f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflection point</td>
<td>60.3% of peak height</td>
<td>4.000</td>
</tr>
<tr>
<td>Half-height</td>
<td>50% of peak height</td>
<td>5.545</td>
</tr>
<tr>
<td>$4\sigma$ method</td>
<td>13.4% of peak height</td>
<td>16.00</td>
</tr>
<tr>
<td>$5\sigma$ method</td>
<td>4.4% of peak height</td>
<td>25.00</td>
</tr>
<tr>
<td>Tangent method</td>
<td>Intersection of tangents with baseline</td>
<td>16.00</td>
</tr>
</tbody>
</table>

PERFORMANCE CRITERIA BY ONE PEAK

RETENTION FACTOR or CAPACITY RATIO

$$k' = \frac{t_R - t_0}{t_0} \quad k' = \phi \frac{C_s}{C_m}$$

APPLICATION

ASYMMETRY FACTOR

$$A_f = \frac{B_{(10\% h)}}{A_{(10\% h)}}$$

TAILING FACTOR

$$T_f = \frac{A + B}{2A}$$

NUMBER OF THEORETICAL PLATES

$$N = 16 \left( \frac{t_R}{W} \right)^2$$
PERFORMANCE BY TWO PEAKS

SELECTIVITY FACTOR

\[ \alpha = \frac{k'(2)}{k'(1)} \]

EXPERIMENTAL RESOLUTION

\[ R_s = \frac{t_{R(2)} - t_{R(1)}}{1/2 (w_1 + w_2)} \]
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Separation Development Sequence

FIRST INJECTION

ADJUST k’ (Retention)

ADJUST N (Plates)

ADJUST alpha (Separation)

Higher Components ratio - Higher Resolution Required

Figure 3: Same as figure 2, except the peak area ratio for the two peaks is 100:1.

(a) 100:1

(b) 10:1

Figure 2: Same as figure 1, except the peak area ratio for the two peaks is 10:1. The peak heights are expanded to aid visualization of the smaller peaks.

(c) 10:1

(d) 10:1

6.6 6.8 7.0 7.2 7.4 Time (min)
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The importance of efficient chromatography

• Higher Sensitivity
• Separation between related compounds
• Higher peak capacity
• Shorter run times
• Solvents and cost saving

SELECTIVITY vs EFFICIENCY

LOW SELECTIVITY \((\alpha)\)  
HIGH EFFICIENCY \((N)\)  

HIGH SELECTIVITY \((\alpha)\)  
LOW EFFICIENCY \((N)\)  

\[ Rs = \frac{t_{R(2)} - t_{R(1)}}{1/2(w_1 + w_2)} \]  
Same in both cases
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**SELECTIVITY vs EFFICIENCY**

$$Rs = \frac{t_R(2) - t_R(1)}{1/2(w_1 + w_2)}$$

**Low Selectivity (α)**

**High Efficiency (N)**

**High Selectivity (α)**

**Low Efficiency (N)**

---

**Fast Gradient Application**

- **Columns:** X Terra™ MS C18 2.1 x 20 mm, 2.5 μm; X Terra™ MS C18 2.1 x 50 mm, 5 μm
- **Mobile Phase:** A = 0.1% TFA in water; B = 0.08% TFA in MeCN
- **Gradient:** 5 - 95% B in 45 seconds and 120 seconds
- **Column Temperature:** 60 °C
- **Flow Rate:** 1.5 mL/min.
- **Detector:** 254 nm
- **Injection Volume:** 1 μL

---

**Fast Isocratic Application**

- **UPLC**
- **Method:** Buffer phosphate pH4
- **Flow:** 0.4 mL/min
- **Temp:** 30C
- **Column Acquity 2.1 x 100 mm**
- **280 nm:** 40 pA/sec
Recommendations Parameters: System Suitability

- Capacity factor
  - $k' > 2$
- Precision/Injection repeatability
  - $\text{RSD} \leq 1\%$, $n \geq 5$
- Resolution
  - $Rs \geq 2$ (Major peak and closest eluting)
- Tailing factor
  - $T \leq 2$
- Theoretical Plates
  - $N \geq 2000$

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### Quality Control – Test of Precision and Calibration

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<th>INJ VOL</th>
<th>No of Inj</th>
<th>Function</th>
<th>Method</th>
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<th>Dilution</th>
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<td>1.00000</td>
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</table>

### Summary Report

**Sample Set:** PrednisoneNoRecircNoRepl

**General Information**
- Sample Set Name: PrednisoneNoRecircNoRepl
- Sample Set Method: PrednisoneProcMth
- Sample Set Start Date: 27/10/1999 12:36:43
- Sample Set Finish Date: 27/10/1999 15:37:29
- Sample Set Id: 2090
- Sample Set Altered: No

**Sample/Label**

<table>
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<tr>
<th>Sample/Label</th>
<th>Sample Type</th>
<th>Vial</th>
<th>Inj #</th>
<th>Run Time (Minutes)</th>
<th>Injection Volume (ul)</th>
<th>Acquisiton Method Set</th>
<th>Sample Weight</th>
<th>Dilution</th>
<th>Label</th>
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</table>

**Calibration Plot**

Name: Prednisone; Processing Method: Prednisone Proc Mth; Fit Type: Linear (1st Order); Cal Curve Id: 2973; A: 0.0000000000; B: 4.867524e+007; C: 0.0000000000; D: 0.0000000000; F 2: 1.0000000000
Quantitative work in HPLC

**Assay**

\[
\frac{ASpl \times WStd \times P}{WSpl \times AStd} = \% \text{ of Impurity}
\]

Legend:
- **ASpl**: Area of impurity in the sample
- **AStd**: Area of standard peak
- **WSpl**: Weight of sample (mg/mL)
- **WStd**: Weight of standard (mg/mL)

**Related Compounds**

\[
\% \text{ Known Impurity} = \frac{Area \ Spl \times Purity \ Std. \times 100}{Concentration \ Spl \times F \ Std}
\]

Legend:
- **Area Spl**: Area of known impurity in the sample.
- **F Std**: Known Impurity Standard Area / Known Standard concentration.
- **Purity Std**: Purity of Known Standard.

The percentage of unknown impurities is calculated by the formula:

\[
\% \text{ Unknown Impurity} = \frac{Area \ Spl \times Purity \ Std. \times 100}{Concentration \ Spl \times F \ Std}
\]

Legend:
- **Area Spl**: Area of unknown impurity in the sample.
- **F Std**: MAIN Standard Area / MAIN Standard concentration.
- **Purity Std**: Purity of MAIN Standard.
Quantitative work in HPLC

**Working Curve**

A plot of the analytical signal (the instrument or detector response) as a function of analyte concentration, using a series of standards of known concentration.

The working curves are then used to determine the concentration of an unknown sample or to calibrate the linearity of an analytical instrument.

**Choice of Standardization:**

**External or Internal**

**External Standard**

\[
\text{Amount Unk} = \frac{\text{Amount Std}}{\text{Response Std}} \times \text{Response Unk}
\]

\[
b = \frac{\sum (x_i - \bar{x})(y_i - \bar{y})}{\sum (x_i - \bar{x})^2}
\]
Choice of Standardization

External or Internal

Simple formulations and sample preparation: external standard

Gas chromatography, bio-studies or complex medium and complex sample preparation: internal standard
Quantitative work in HPLC

**Internal Standard**

\[
\frac{12,000}{6000} \rightarrow 100/10 \quad \text{and} \quad \frac{11,000}{5800} \rightarrow \text{Amt B/10}
\]

\[
(11,000/5,800) \times (100/10) \quad \text{----------------------------------- x} \quad 10 = \text{Amt B} = 94.8
\]

**Standard Addition**

Due to matrix effects, the analytical response for an analyte in a complex sample may not be the same as for the analyte in a simple standard.
Quantitative work in HPLC

Summary Report
Sample Set: PrednisoneNoRecircNoRepl

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Sample Type</th>
<th>Vial</th>
<th>Inj #</th>
<th>Run Time (Minutes)</th>
<th>Injection Volume (ul)</th>
<th>Acquisition Method Set</th>
<th>Sample Weight</th>
<th>Dilution</th>
<th>Label</th>
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<tbody>
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Calibration Plot

Name: Prednisone; Processing Method: Prednisone Proc Mth; Fit Type: Linear (1st Order); Cal Curve Id: 2973; A: 0.000000e+000; B: 4.967524e+007; C: 0.000000e+000; D: 0.000000e+000; R^2: 1.000000

Standard Deviation

- A measure of the uncertainty due to random error in a set of data (also: precision of a set of measurements).

\[
s = \sqrt{\frac{1}{N-1} \sum_{i=1}^{N} (x_i - \bar{x})^2}
\]

\[s = \text{standard deviation},\]
\[N = \text{the number of data points},\]
\[x_i = \text{each individual measurement},\]
\[\bar{x} = \text{the mean of all measurements}.

The value \(x_i - \bar{x}\) is called the residual for each measurement.
### Quality Control – Test of Accuracy

<table>
<thead>
<tr>
<th>VIAL</th>
<th>SAMPLE NAME</th>
<th>INJ VOL</th>
<th>No of Inj</th>
<th>Function</th>
<th>Method</th>
<th>Run Time</th>
<th>Sample Weight</th>
<th>Dilution</th>
<th>Weight</th>
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### Control – Std2 (Accuracy/Recovery Test)

#### Summary of Standards

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<th>Dose Microgram</th>
<th>VIAL</th>
<th>IE</th>
<th>IE%</th>
<th>AUE</th>
<th>SUE</th>
<th>SPE</th>
<th>RPE</th>
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### % Difference between Standards

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Quantitative work in HPLC

### Standards Comparison

**Name:** methyl paraben

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<th>Vial</th>
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<th>Area</th>
<th>Amount Area_Amount</th>
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<tbody>
<tr>
<td>std-1</td>
<td>S1 methyl paraben</td>
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<td>2.775</td>
<td>7208895</td>
<td>44.429</td>
<td>179469</td>
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<td>std-1</td>
<td>S1 methyl paraben</td>
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<td>2.775</td>
<td>7208895</td>
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### Control Summary Table

**Name:** methyl paraben

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<th>Vial</th>
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<tbody>
<tr>
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<td>2.783</td>
<td>1598421</td>
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<td>2.783</td>
<td>1598421</td>
<td>44.88</td>
<td>44.857</td>
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### Standards Summary Table

**Name:** propyl paraben

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<th>Amount Area_Amount</th>
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<td>std-1</td>
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<td>3.054</td>
<td>679529</td>
<td>45.014</td>
<td>14999</td>
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<td>std-1</td>
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<td>3.054</td>
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<td>S1 propyl paraben</td>
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<td>3.054</td>
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<td>45.014</td>
<td>14999</td>
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<td>std-1</td>
<td>S1 propyl paraben</td>
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<td>45.014</td>
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<td>Mean</td>
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### Control Summary Table

**Name:** propyl paraben

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<th>RT</th>
<th>Area</th>
<th>Amount Area_Amount</th>
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</thead>
<tbody>
<tr>
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<td>S2 propyl paraben</td>
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<td>2.799</td>
<td>7097963</td>
<td>44.88</td>
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<tr>
<td>std-2</td>
<td>S2 propyl paraben</td>
<td>1</td>
<td>2.799</td>
<td>7097963</td>
<td>44.88</td>
<td>44.857</td>
</tr>
</tbody>
</table>

---

### Determination of a Control Standard:

**Test of Accuracy**

- Amount Std ➔ Response Std
- Amount Control ➔ Response Unk

\[
\text{Amount Control} = \frac{\text{Amount Std \ Response Std}}{\text{Response Unk}}
\]

**Accuracy:**

\[
\frac{\text{Amount Control Determined}}{\text{Amount Control Prepared}} = 0.99 - 1.01
\]

---

Reported by User: System
Report Method: Standards_Comparison_Multi
Project Name: Pharma QC calculations_Trima
Report Method ID: 4920
Date Printed: 16/04/2010
21:02:38 Asia/Tel_Aviv
### Running the Unknowns

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<th>No of Inj</th>
<th>Function</th>
<th>Method</th>
<th>Run Time</th>
<th>Sample Weight</th>
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<tbody>
<tr>
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### Quality Control - Quantification

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<th>VIAL</th>
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<th>No of Inj</th>
<th>Function</th>
<th>Method</th>
<th>Run Time</th>
<th>Sample Weight</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
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</tr>
</tbody>
</table>

Report: LC Calibration Report
Report: Standard Comparison
Clear Calibration: LC Demo Method Set
Basic Report

SAMPLE INFORMATION

Sample Name: Prednisone_Tab
Sample Type: Unknown
Vial: 16
Injection #: 1
Injection Volume: 20.00 ul
Run Time: 4.0 Minutes
Sample Set Name: PrednisoneNoRecircNoRep

Acquired By: System
Date Acquired: 27/10/1999 14:38:40
Acq. Method Set: Prednisone Mth Set
Vial: 16
Injection Volume: 20.00 ul
Run Time: 4.0 Minutes
Sample Set Name: PrednisoneNoRecircNoRep

Date Processed: 27/03/2001 14:17:26
Processing Method: Prednisone Proc. Mth
Channel Name: 2487/Channel 1
Proc. Chnl. Descr.: Single @ 242 nm

Auto-Scaled Chromatogram

Peak Results

<table>
<thead>
<tr>
<th>Name</th>
<th>RT</th>
<th>Area</th>
<th>Height</th>
<th>Amount</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prednisone</td>
<td>0.573</td>
<td>22375</td>
<td>822</td>
<td>22575</td>
<td>1188824</td>
</tr>
</tbody>
</table>

Calibration Procedures
Quantitative work in HPLC

- Standards Considerations
- Integration events: Explained and demonstrated
- Running sets of samples
  - Blanks – Carry over and system peaks
  - System Suitability
  - Calibration and Quantitation
- Parameters effecting quantitation and validation
- Requirements from chromatographic data systems

Parameters To Monitor - Validation

- Precision (Ruggedness)
- Accuracy
- Limit of detection
- Limit of quantitation
- Linearity (range)
- Selectivity
- Robustness
Example Method Validation Protocol

Parameters To Monitor - Validation

- Precision (Ruggedness)
- Accuracy
- Limit of detection
- Limit of quantitation
- Linearity (range)
- Selectivity
- Robustness
Quantitative work in HPLC

CHARACTERISTICS OF AN ANALYTICAL METHODS

Precision: The reproducibility of results. The degree to which an experimental result varies from one determination to the next.

Precision is related to random error and Accuracy is related to systematic error.

Illustrating the difference between “accuracy” and “precision”

- Low accuracy, low precision
- Low accuracy, high precision
- High accuracy, low precision
- High accuracy, high precision

Precision: Definition

- Precision
  - The measure of the degree of agreement among test results when the method is applied repeatedly to multiple samplings of a homogeneous sample
  - Expressed as %RSD for a statistically significant number of samples
Precision: Definition

- Precision Should Be Performed at Three Levels
  - Repeatability
  - Intermediate Precision
  - Reproducibility

Precision: Definition/Determination

- **Repeatability** (Generally the criterion of concern in USP analytical procedures)
  - Same operating conditions, short time interval
  - Intra-assay precision
    - Minimum of 9 determinations covering specified range of procedure (3 levels, 3 reps each), or
    - Minimum of 6 determinations at 100% test conc.
- **Intermediate Precision** (Experimental design recommended)
  - Within-lab variations (Random events)
  - Different days, analysts, equipment
- **Reproducibility**
  - Precision between labs
  - Collaborative studies
**Ruggedness: Definition**

- **Ruggedness**
  - Degree of Reproducibility of Test Results Under a Variety of Conditions
    - Different Laboratories
    - Different Analysts
    - Different Instruments
    - Different Reagents
    - Different Days
    - Etc.
  - Expressed as %RSD

**Calculating Precision**

\[
\text{Mean} : \quad \bar{X} = \frac{\sum_{i=1}^{N} X_i}{N}
\]

\[
\text{Standard Deviation} : \quad \sigma = \sqrt{\frac{\sum_{i=1}^{N} (X_i - \bar{X})^2}{N - 1}}
\]

\[
\text{Coefficient of Variation} : \quad CV\% = \frac{\sigma}{\bar{X}} (100)
\]
### Example Precision Data

<table>
<thead>
<tr>
<th>Injection</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>125955</td>
</tr>
<tr>
<td>2</td>
<td>125651</td>
</tr>
<tr>
<td>3</td>
<td>126052</td>
</tr>
<tr>
<td>4</td>
<td>124855</td>
</tr>
<tr>
<td>5</td>
<td>126102</td>
</tr>
<tr>
<td>6</td>
<td>125899</td>
</tr>
<tr>
<td>Average</td>
<td>125752</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>466.93</td>
</tr>
<tr>
<td>Relative Standard Deviation</td>
<td>0.37%</td>
</tr>
</tbody>
</table>

### Precision Test

![Precision Test Chart]

**Component Summary Table**

<table>
<thead>
<tr>
<th>Component</th>
<th>Name</th>
<th>RT</th>
<th>Area</th>
<th>Height</th>
<th>Amount</th>
<th>Vial</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SysSul_A</td>
<td>1</td>
<td>2487</td>
<td>Channel 1</td>
<td>1</td>
<td>E1F</td>
</tr>
<tr>
<td>2</td>
<td>SysSul_A</td>
<td>2</td>
<td>2487</td>
<td>Channel 1</td>
<td>1</td>
<td>E1F</td>
</tr>
<tr>
<td>3</td>
<td>SysSul_A</td>
<td>3</td>
<td>2487</td>
<td>Channel 1</td>
<td>1</td>
<td>E1F</td>
</tr>
<tr>
<td>4</td>
<td>SysSul_A</td>
<td>4</td>
<td>2487</td>
<td>Channel 1</td>
<td>1</td>
<td>E1F</td>
</tr>
<tr>
<td>5</td>
<td>SysSul_A</td>
<td>5</td>
<td>2487</td>
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<td>1</td>
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</tr>
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<td>SysSul_A</td>
<td>6</td>
<td>2487</td>
<td>Channel 1</td>
<td>1</td>
<td>E1F</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Std Dev vs RSD**

- Std Dev: 1.177
- RSD: 0.10
- 0.40
Quantitative work in HPLC

Documenting Precision With Bar Charts

Precision - Acceptance Criteria

- Less than 2% relative standard deviation is often recommended.
- Less than 5% RSD can be acceptable for minor components.
- Up to 10% RSD may be acceptable near the limit of quantitation.
CHARACTERISTICS OF AN ANALYTICAL METHOD

Accuracy: The degree to which an experimental result approaches the true or accepted answer.

Ways to Describe Accuracy:

Error: An experimental measure of accuracy. The difference between the result obtained by a method and the true or accepted value.

\[ \text{Absolute Error} = (X - \mu) \]
\[ \text{Relative Error} (%) = 100(X - \mu)/\mu \]

where: \( X \) = The experimental result
\( \mu \) = The true result

Two types of error: random or systematic

ERRORS

Random Error: results in a scatter of results centered on the true value for repeated measurements on a single sample. (SPREAD)

Systematic Error: results in all measurements exhibiting a definite difference from the true value (BIAS)

plot of the number of occurrences or population of each measurement (Gaussian curve)
CHARACTERISTICS OF AN ANALYTICAL METHOD

Ways to Describe Precision:

Range: the high to low values measured in a repeat series of experiments.

Standard Deviation: describes the distribution of the measured results about the mean or average value.

Absolute Standard Deviation (SD):

\[ SD = \sqrt{\frac{\sum_{i=1}^{n} (X_i - \bar{X})^2}{(n-1)}} \]

Relative Standard Deviation (RSD) or Coefficient of Variation (CV):

\[ RSD \% = \left( \frac{SD}{\bar{X}} \right) \times 100 \]

where:  
- \( n \) = total number of measurements  
- \( X_i \) = measurement made for the ith trial  
- \( \bar{X} \) = mean result for the data sample

Overlaid Chromatograms

Good Precision

![Overlaid Chromatograms](chart)

SampleName 2690_Eau40_60CH3OH_2mm_D Vial 4 Injection 1
SampleName 2690_Eau40_60CH3OH_2mm_D Vial 4 Injection 2
SampleName 2690_Eau40_60CH3OH_2mm_D Vial 4 Injection 3
SampleName 2690_Eau40_60CH3OH_2mm_D Vial 4 Injection 4
SampleName 2690_Eau40_60CH3OH_2mm_D Vial 4 Injection 5
SampleName 2690_Eau40_60CH3OH_2mm_D Vial 4 Injection 6
Quantitative work in HPLC

Parameters To Monitor - Validation

- Precision (Ruggedness)
- Accuracy
- Limit of detection
- Limit of quantitation
- Linearity (range)
- Selectivity
- Robustness

Method Transfer - 3 different HPLC Systems
Quantitative work in HPLC

**Column Batch-to-Batch Reproducibility**

- **Batch 108**
- **Batch 109**
- **Batch 112**

Sample: AZT
Injection: 150 µL of 0.5 mg/mL solution
Column: Symmetry C18, 3.9 mm x 150 mm
Temperature: 45 °C
Mobile Phase: 6% MeOH/6% THF/88%
10 mM potassium phosphate buffer, pH 2.5
Flow rate: 1.7 mL/min
Detector: UV at 268 nm

**Factors Affecting Precision**

**Area and Retention Time**

- Integration parameters
- Injector performance
- Pump performance
Quantitative work in HPLC

Variable Reported Concentrations
Problems with Peak Response

Linearity Test of Concentrations

- **Check Injector** (Use Standards)
  * Multiple Injections - Same Vial -- Syringe Problem
  * If Only 1st Injection Low -- Septa Problem
  * Different Vials -- Evaporation -- Degradation
  * Injection Volume Test (Weight before and after injection)

- **Integration Software**
  * Electronic Peak Generator
  * Poor Peak Shape

- **Detector**
  * Cell Problem
  * Lamp Failing

- **Detector**
  * Cell Problem
  * Lamp Failing

**Incorporate a graph showing the relationship between injection volume and response, with R² = 0.9999.**

**Incorporate a graph showing RSD < 5-15%.**

**Incorporate a graph showing different volumes of the same standard.**

**Incorporate a graph showing methods transfer.**
Quantitative work in HPLC

Accuracy: Definition

- The closeness of test results obtained by the method to the true value.
  - Established across the concentrations range

Accuracy: Determination

- **Bio-active Substance**
  - Analysis of reference material
- **Bio-active Formulation**
  - Analysis of synthetic mixtures spiked with known quantities of components
- **Impurities (Quantitation)**
  - Analysis of samples (Bio-active substances/Bio-active formulation) spiked with known amounts of impurities
  - If impurities are not available, see specificity
**Accuracy: Determination (Cont.)**

- **Recommended Data**
  - Minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g. 3 concentrations/3 replicates each)
  - Reported as % recovery of known, added amount, or difference between the mean and true value, with confidence intervals

---

**Accuracy of Measurement**

Correlation Coefficient $r$ for $(x_i, y_i)$

$$r = \frac{\sum (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum (x_i - \bar{x})^2 \sum (y_i - \bar{y})^2}}$$

- $x_i, \bar{x}$ from method one
- $y_i, \bar{y}$ from method two

**Error**

$E = O - T$

$O = $ Observed Value

$T = $ True Value

**% Relative Error**

$$%RE = \frac{E}{T} \times 100$$
Example Accuracy Data

<table>
<thead>
<tr>
<th>Target Concentration</th>
<th>Spiked Concentration (mg/mL)</th>
<th>Measured Concentration (mg/mL) Avg N=3</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>50%</td>
<td>0.50</td>
<td>0.49</td>
<td>98.0</td>
</tr>
<tr>
<td>75%</td>
<td>0.75</td>
<td>0.74</td>
<td>98.7</td>
</tr>
<tr>
<td>100%</td>
<td>1.00</td>
<td>1.03</td>
<td>103</td>
</tr>
<tr>
<td>125%</td>
<td>1.25</td>
<td>1.24</td>
<td>99.2</td>
</tr>
<tr>
<td>150%</td>
<td>1.50</td>
<td>1.48</td>
<td>98.7</td>
</tr>
</tbody>
</table>

Average Recovery: 99.52%
Standard Deviation: 1.99
Relative Standard Deviation: 2.00

Documenting Accuracy with Control Charts

Custom Report Control Chart for: ACE, Channel: 996

Run Number

Area

Target

UCL

LCL
Quantitative work in HPLC

Parameters To Monitor - Validation
(See Detection Qualification)

Limit of Detection: \( h_{\text{signal}} = 2 \times h_{\text{noise}} \)
Limit of Quantitation: \( h_{\text{signal}} = 10 \times h_{\text{noise}} \)

- Precision (Ruggedness)
- Accuracy
- Limit of detection
- Limit of quantitation
- Linearity (range)
- Selectivity
- Robustness

Detection Limit (DL/LOD): Determination

- Visual (Non-Instrumental Methods)
- Signal To Noise Ratio (3 or 2:1 Generally Accepted)
- Detection limit may be based on the standard deviation of the response and slope:

\[
DL = (3.3)STD/S
\]

STD = standard deviation of the response
S = slope of the calibration curve
Quantitative work in HPLC

Quantitation Limit (QL/LOQ): Definition

- **Limit of Quantitation (LOQ)**
  - Lowest Concentration of Analyte in a Sample That Can Be Determined With Acceptable Precision and Accuracy Under Stated Operational Conditions
  - Expressed as the Concentration of Analyte
    - Accuracy
    - Precision

Quantitation Limit (QL/LOQ): Determination

- Visual (Non-Instrumental Methods)
- Signal To Noise Ratio (10:1 is Typical)
- Quantitation limit may be based on the standard deviation of the response and slope:

\[
QL = (10)STD/S
\]

STD = standard deviation of the response
S = slope of the calibration curve

Both DL and QL are validated by analyzing a suitable number of samples. Method should be documented
Example LOQ/LOD Data and Calculations

<table>
<thead>
<tr>
<th>Level 1 Response</th>
<th>Level 2 Response</th>
<th>Level 3 Response</th>
<th>Level 4 Response</th>
<th>Level 5 Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2085</td>
<td>4105</td>
<td>6288</td>
<td>8333</td>
</tr>
<tr>
<td>2</td>
<td>2106</td>
<td>4235</td>
<td>6103</td>
<td>8287</td>
</tr>
<tr>
<td>3</td>
<td>1955</td>
<td>4188</td>
<td>6337</td>
<td>8399</td>
</tr>
<tr>
<td>4</td>
<td>2199</td>
<td>4063</td>
<td>6129</td>
<td>8156</td>
</tr>
<tr>
<td>5</td>
<td>2077</td>
<td>4005</td>
<td>6254</td>
<td>8247</td>
</tr>
<tr>
<td>6</td>
<td>2155</td>
<td>4284</td>
<td>6301</td>
<td>8178</td>
</tr>
</tbody>
</table>

Mean     2096  4147  6235  8267  10371  
STDev.  83.13 106.9 96.53 92.54 102.57  

Average Standard Deviation:  96.33  
Slope of Calibration Curve:  9378  
Limit of Detection:  0.034 mg/mL  
Limit of Quantitation:  0.103 mg/mL  

Effect of HPLC Columns’s Performance in Method Validation: LOQ

System Suitability results of Tailing and Plate Count would have predicted the differences

Sample: 0.25 µg/mL Tamoxifen
Injection volume and linear flow velocity compensated for on both columns
Parameters To Monitor - Validation

- Precision (Ruggedness)
- Accuracy
- Limit of detection
- Limit of quantitation
- Linearity (range)
- Selectivity
- Robustness

Linearity and Range: Definition

- **Linearity**
  - The Ability of the Method to Elicit Test Results That Are Directly Proportional to Concentration Within a Given Range
  - Expressed as the Variance of the Slope of the Regression Line

- **Range**
  - Interval between upper and lower levels of analyte demonstrated by the method
  - Precision and Accuracy Expressed in the same units as the test results
Quantitative work in HPLC

Linearity: Determination

- **Established across the Range of the method**
  - Dilutions
  - Separate Weighings
- **Evaluate by Appropriate Statistical Methods** (e.g. Regression)
  - Include Correlation Coefficient, y-Intercept, Slope, Residual Sum of Squares, Plot Itself
- **Minimum 5 Concentrations**

Determination of Appropriate Range

- **Minimum Specified Ranges**
  - Assay
    - 80-120%
  - **Impurity Test**
    - From QL to 120% of spec.
    - Toxic or more potent impurities: commensurate with the controlled level
  - **Content Uniformity**
    - 70-130% of test concentration
- **Dissolution Testing**
  - +/- 20% over specified range
Example linearity (Calibration) Plot

Linearity (Calibration) Plot

Include correlation coefficient, y-intercept, slope, plot itself
Minimum of five concentrations, +/- 20% over specified range
Quantitative work in HPLC

Example for HPLC Method Precision

Repeatability:  N = 6 at 100%

<table>
<thead>
<tr>
<th>Day</th>
<th>%RSD-Time</th>
<th>%RSD-Time</th>
<th>%RSD-Area</th>
<th>%RSD-Area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCT</td>
<td>TMT</td>
<td>HCT</td>
<td>TMT</td>
</tr>
<tr>
<td>1</td>
<td>0.00</td>
<td>0.00</td>
<td>0.05</td>
<td>0.06</td>
</tr>
<tr>
<td>2</td>
<td>0.05</td>
<td>0.00</td>
<td>0.16</td>
<td>0.07</td>
</tr>
<tr>
<td>3</td>
<td>0.00</td>
<td>0.00</td>
<td>0.18</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Intermediate Precision:
N = 18 (6 inj./day, 3 days) at 100%

<table>
<thead>
<tr>
<th>Compound</th>
<th>%RSD-Time</th>
<th>%RSD-Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT</td>
<td>0.21</td>
<td>3.24</td>
</tr>
<tr>
<td>TMT</td>
<td>0.56</td>
<td>3.97</td>
</tr>
</tbody>
</table>

Parameters To Monitor - Validation

- Precision (Ruggedness)
- Accuracy
- Limit of detection
- Limit of quantitation
- Linearity (range)
- Selectivity/Specificity
- Robustness
Specificity: Definition

- **Specificity (Selectivity)**
  - The ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the matrix
  - The degree of interference
    - Active Ingredients
    - Excipients
    - Impurities
    - Degradation Products
    - Placebo Ingredients

Specificity: Determination

- **Qualitative Identification Tests**
  - Demonstrate ability to select between compounds of closely related structure

- **Assay**
  - Demonstrate that the results are unaffected by spiked impurities or excipients

- **Impurities**
  - Spike the drug product/substance with impurities and demonstrate appropriate accuracy and precision
Method Development Challenge

- Excipients
- Impurities
- Degradants

Composite sample

Courtesy of:
Dr. Rudy Sneyers, J&J Belgium

PDA is used mostly for Development and Validation of STABILITY INDICATING METHODS
What is a Stability Indicating Method?

- A Stability Indicating Method (SIM) is:
  - A *validated* method that can accurately and precisely quantitate the decrease of the API content due to degradation.
  - **The method:**
    - Is *specific* for the drug substance
    - Shows a *decrease* in assay value (correlated to drug substance loss) due to degradation
    - Has no *interference* from excipients, impurities or degradation products
    - Detects and quantitates impurities and degradation products

Why are Stability Indicating Methods Needed?

Why are Stability Indicating Methods needed?
Predominantly used to support long-term stability testing
- How the quality of the drug substance or product changes over time in response to environmental factors
  - Temperature
  - Humidity
  - Light
  - Establishes storage and packaging conditions
When to Use
Stability Indicating Methods

• When are SIMs needed?
  – Stability studies
  – API release
  – Drug product release
  – Toxicology dosing solutions
  – Excipient compatibility and pre-formulation
  – Packaging studies
  – Line extensions

• When are SIMs not needed?
  – In process controls
  – Secondary assay for API
    • Titration
  – Inorganics

Sample Generation
Forced Degradation (Stress) Studies

• Forced degradation or stress testing is undertaken to demonstrate specificity when developing SIMs
  – Generates a sample for method development

• Performed prior to implementation of stability studies

• Why do an intentional degradation study?
  – Understand the reactive chemistry of the drug substance
  – Help anticipate future stability issues of both drug substance and drug product
  – Provides useful information for formulation and stability
  – May be required for regulatory submissions
Sample Generation

- Common conditions used in Forced Degradation Studies

<table>
<thead>
<tr>
<th>Study</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic pH</td>
<td>0.1N HCl</td>
</tr>
<tr>
<td>Neutral pH</td>
<td>pH 7.0 Phosphate Buffer</td>
</tr>
<tr>
<td>Basic pH</td>
<td>0.1N NaOH</td>
</tr>
<tr>
<td>Oxidation</td>
<td>$O_2$ Atmosphere, or $H_2O_2$</td>
</tr>
<tr>
<td>Photolysis (UV)</td>
<td>1000 Watt h/M2</td>
</tr>
<tr>
<td>Photolysis (Fluorescence)</td>
<td>$6 \times 10^6$ lux h</td>
</tr>
</tbody>
</table>

Goal: Degrade API 5-10%

Stability Indicating Method Development

- Manipulate chromatographic selectivity
  - Column
  - Mobile phase composition/type
  - pH
  - Temperature
- Specificity
  - PDA or MS

Goal: Baseline Resolution, No Co-elutions
Quantitative work in HPLC

More Changes Courtesy of ICH: Specificity

PDA and MS Together

PDA and MS together can provide complimentary useful information.
Quantitative work in HPLC

Diode Array Detector

Principle of Measurement

3 dimensional plot of a chromatographic run using a diode-array detector
Quantitative work in HPLC

Typical LC/MS System Components

SOURCE

HPLC

LC/MS INTERFACE

MASS SPECTRUM SORTING OF IONS

ANALYZER

ION DETECTOR

DETECTION OF IONS

SORTING OF IONS

SAMPLE DESOLVATION AND IONIZATION

DATA SYSTEM

3 dimentional plot of a chromatographic run
using a mass spectrometer detector
Quantitative work in HPLC

Chromatogram of three sulfa drugs at 270 nm and a plot of UV Spectra collected from each peak's Apex, Inflection and two offset points, to demonstrate comparison of spectra collected at various points across the peak.

SIR chromatograms of the same sulfa drugs and their corresponding mass spectra, obtained by combining spectra across the entire peak.
Quantitative work in HPLC

MS Sensitivity And Specificity Example

Impurity study of Lansoprazole : UV detection

Lansoprazole
UV @ 254nm

MS Sensitivity And Specificity Example

Impurity study of Lansoprazole : MS detection (SIR mode)
MW+1= 298.2

Lansoprazole
UV @ 254nm
SIR @ 298.2 m/z
Sample Generation

- Common conditions used in Forced Degradation Studies

<table>
<thead>
<tr>
<th>Study</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic pH</td>
<td>0.1N HCl</td>
</tr>
<tr>
<td>Neutral pH</td>
<td>pH 7.0 Phosphate Buffer</td>
</tr>
<tr>
<td>Basic pH</td>
<td>0.1N NaOH</td>
</tr>
<tr>
<td>Oxidation</td>
<td>O₂ Atmosphere, or H₂O₂</td>
</tr>
<tr>
<td>Photolysis (UV)</td>
<td>1000 Watt h/M2</td>
</tr>
<tr>
<td>Photolysis (Fluorescence)</td>
<td>6x10⁶ lux h</td>
</tr>
</tbody>
</table>

Goal: Degrade API 5-10%
Quantitative work in HPLC

Forced Degradation Results

**UV-VIS**
- Simvastatin
- Acid Hydrolysis
- Thermal Degradation
- Peroxide Oxidation
- Photo Degradation (Dry Powder)
- Photo Degradation (Solution)
- Base Hydrolysis

**Mass Spectrometry**
- Simvastatin
- Acid Hydrolysis
- Thermal Degradation
- Peroxide Oxidation
- Photo Degradation (Dry Powder)
- Photo Degradation (Solution)
- Base Hydrolysis

Specificity: Determination (Cont.)

- **Impurities Are Available**
  - Demonstrate that the assay is unaffected by the presence of spiked materials (impurities and/or excipients).

- **Impurities Are Not Available**
  - Compare test results to a second well-characterized procedure
    - For Assay, compare the two results
    - For Impurity Tests, compare impurity profiles
  - Peak Purity Test ("diode array, MS")
Quantitative work in HPLC

**MS Detection in Peak Purity Analyses**

Enhance 3D PDA information with mass spec information:

- Added confidence in methods development (peak confirmation)
- Peak homogeneity (detect co-eluting compounds)
- Peak tracking
- Enhanced sensitivity
- Enhanced selectivity
- Non-UV absorbing compounds
  - Contributes to “universal detection” concept

**Parameters To Monitor - Validation**

- Precision (Ruggedness)
- Accuracy
- Limit of detection
- Limit of quantitation
- Linearity (range)
- Selectivity
- Robustness
Robustness: Definition

- Robustness
  - Measure of The Capacity to Remain Unaffected by Small (Deliberate) Variations in Method Parameters
  - Indication of Reliability During Normal Use

Robustness: Determination

- Consider during development of method
- Shows reliability of method with respect to deliberate changes
- If measurements are susceptible to variations in analytical procedures, these conditions should be controlled and a precautionary statement included.
- Establish System Suitability parameters to ensure the validity of the method
Robustness of the chromatographic method

- Parameters Varied:
  - Solvent strength in the mobile phase,
  - Temperature,
  - Flow rate,
  - pH of the mobile phase,
  - Ionic strength in the mobile phase,
  - Sample diluent,
  - Injection volume,
  - Wavelength of detection.

The parameter measured:
- Response (area/amount)
- Retention time,
- Selectivity and/or resolution.

Non-Robust Method

pH=4.9

pH=4.5

Minor Changes in pH Effect Rs

pH=4.7
Quantitative work in HPLC

Robust Method

**AZT: Robustness Testing**

6% Methanol, 6% THF

- **pH 2.3**
- **pH 2.5**
- **pH 2.7**

Method Change Versus Adjustment Proposals

- **Aqueous Buffer pH**
  - Analytes without ionizable groups: +/- 1 unit
  - Analytes with basic or acidic groups and the buffer pH = pKa +/- 2 units: +/- 0.2 unit
  - Analytes with basic or acidic groups and the buffer pH < or > pKa +/- 2 units: +/- 1 unit
    - Column Dimensions
      - Length: +/- 70%
      - Inner Diameter: +/- 25%, maintaining a constant linear velocity
      - Flow Rate: up to 50%

Reference standards must be used to show that the chromatography is improved by pH adjustment.
Quantitative work in HPLC

- Standards Considerations
- Integration events: Explained and demonstrated
- Running sets of samples
  - Blanks – Carry over and system peaks
  - System Suitability
  - Calibration and Quantitation
- Parameters effecting quantitation and validation
  - Requirements from chromatographic data systems

Requirements from Chromatographic Data System (CDS)
Quantitative work in HPLC

Basic Sequence in HPLC

1. Login.
2. Instrument Method.
3. Sequence/Run Samples.
5. Review/Preview.

Create a Sequence or a Sample Set/List
Check: Created vs. Accomplished
Quantitative work in HPLC

Create and Use Methods

- Method Set
  - Instrumental parameters
  - Processing parameters
  - Reporting
  - Exporting

Instrumental Parameters - Collect Chromatographic Data

- Method Set
  - Instrumental parameters
  - Processing parameters
  - Reporting
  - Exporting
Quantitative work in HPLC

Example: Instrumental Parameters

Instrument Method : 50B_flow1

Method Information
Method Comments
Method Modified User: System
Method Locked: No
Method Id: 3787
Method Version: 4
Method Edit User:

Pump&Autosampler Instrument Setup
Type
Instrument Status On
Channel Name: 2690/5
Description
Use Channel Monitor Off
Monitor Parameter: System Pressure
Stroke Volume 50uL (flow rates <= 1.23 mL/min)
Stroke Rate
Simulate Draw Rate Normal
Depth Of Needle 0.0
Degas Mode On
Pump Mode: Isocratic
Flow 1.000
%A 50.0
%B 50.0
%C 0.0
%D 0.0
High Limit
Low Limit
Enable Sample Temp False
Enable Column Temp False
Bubble Detect True
Pre Column Volume 0.0

Detector Information
Channel Name
Use Channels: On
Wavelength: 254
Output Mode: Absorbance (ACh1)
Data Mode: Absorbance (ACh1)
Sampling Rate: 2
Filter Type: Hamming

Source S/W Info Empower 2 Software Build 2154 DB ID: 689194452

Process the Data to Create Results
Processing Method

- Method Set
  - Instrumental parameters
  - Processing parameters
  - Reporting
  - Exporting
Quantitative work in HPLC

Processing Method’s Parameters

- Method Set
  - Instrumental parameters
  - Processing parameters
  - Reporting
  - Exporting
Quantitative work in HPLC

### Processing Method Parameters: Example

**Processing Method**
- Type: LC
- Stored: 13/07/2006 14:32:15 EDT

**Method Information**
- Method Comments
- Method Modified User
- Method Locked
- Method Id: 2193
- Method Version: 12
- Method Edit User
- Source S/W Info
- Integration Algorithm: ApexTrack
- Average By: None
- RT Window %: 5.00
- Update RT: Never
- CCPar1
- Include Internal Standard Amounts in Amount Calculations
- Unit/Default/Value Type
- Amount

**System Suitability Information**
- Void Volume Time: 1.500(min)
- Calculate Suitability: Yes
- Calculate: ApexTrack
- Calculate: Yes
- Calculate: All
- Tangent Percent (USP Plate Count): 61
- Tangent Percent (USP Resolution): 50
- Calculate Unknowns: Yes
- Pharmacopeia: All
- Tangent Percent (USP Plate Count): 61
- Calculate: Yes
- Noise Value for s/n: Baseline Noise
- % Runtime: 5.0
- Maximum Drift

**Integration Parameters**
- Minimum Area: 0.000(µV *sec)
- Minimum Height: 0.000(µV)
- Integration Start: 2.000(min)
- Integration End: 11.420(min)
- Peak Width: 11.420(sec)
- Detection Threshold: 5.000e+002(µV)
- Liftoff %: 0.000(%)
- Touchdown %: 0.500(%)
- Baseline Start: 0.000(min)
- Baseline End: 0.000(min)

**Component Table**

<table>
<thead>
<tr>
<th>Name</th>
<th>Component Type</th>
<th>Validation</th>
<th>Target Peak Label</th>
<th>Retention Time</th>
<th>RT Window</th>
<th>Channel</th>
<th>Peak Mode</th>
<th>Calculate Suit Results</th>
<th>Flag Outside Limits</th>
<th>Y Value</th>
<th>X Value</th>
<th>Fit</th>
<th>Weighting</th>
<th>Linear</th>
<th>RT Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 MeP</td>
<td>Main Component</td>
<td>0.000</td>
<td>Closed</td>
<td>Yes</td>
<td>Yes</td>
<td>Area</td>
<td>Amount</td>
<td>Linear</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 EtP</td>
<td>Low Level Impurity</td>
<td>0.000</td>
<td>Closed</td>
<td>Yes</td>
<td>Yes</td>
<td>Area</td>
<td>Amount</td>
<td>Linear</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 PrP</td>
<td>Low Level Impurity</td>
<td>0.000</td>
<td>Closed</td>
<td>Yes</td>
<td>Yes</td>
<td>Area</td>
<td>Amount</td>
<td>Linear</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Report Method**
- Method Set
  - Instrumental parameters
  - Processing parameters
- Reporting
- Exporting
Basic Report

Sample Information

Sample Name: Prednisone_Tab
Sample Type: Unknown
Vial: 15
Injection #: 1
Injection Volume: 20.00 µl
Run Time: 4.0 Minutes
Sample Set Name: PrednisoneNoRecircNoRepl

Acquired By: System
Date Acquired: 27/10/1999 14:38:40

Date Processed: 27/03/2001 14:17:26
Channel Name: 2487Channel 1
Proc. Chnl. Descr.: Single @ 242 nm

Auto-Scaled Chromatogram

Peak Results

<table>
<thead>
<tr>
<th>Name</th>
<th>RT</th>
<th>Area</th>
<th>Height</th>
<th>Amount</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prednisone</td>
<td>0.573</td>
<td>22375</td>
<td>922</td>
<td>0.024</td>
<td>mg/ml</td>
</tr>
</tbody>
</table>

Summary Report

Sample Set: PrednisoneNoRecircNoRepl

Summary Report

Sample Set Name: PrednisoneNoRecircNoRepl
Sample Set Method: PrednisoneNoRecircNoRepl
Sample Set Start Date: 27/10/1999 12:36:43
Sample Set Finish Date: 27/10/1999 15:37:29
Sample Set Id: 2090
Sample Set Altered: No

Sample Name| Sample Type| Vial| Inj #| Run Time (Minutes)| Injection Volume (µl)| Acq. Method Set| Sample Weight| Dilution| Label |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Prednisone</td>
<td>Tab</td>
<td>11</td>
<td>1</td>
<td>4.00</td>
<td>20.00</td>
<td>Prednisone Mth Set</td>
<td>1.00000</td>
<td>1.00000</td>
<td>AU0003</td>
</tr>
<tr>
<td>Prednisone</td>
<td>Tab</td>
<td>10</td>
<td>1</td>
<td>4.00</td>
<td>20.00</td>
<td>Prednisone Mth Set</td>
<td>1.00000</td>
<td>1.00000</td>
<td>AU0004</td>
</tr>
<tr>
<td>Prednisone</td>
<td>Tab</td>
<td>16</td>
<td>1</td>
<td>4.00</td>
<td>20.00</td>
<td>Prednisone Mth Set</td>
<td>1.00000</td>
<td>1.00000</td>
<td>AU0005</td>
</tr>
</tbody>
</table>

Calibration Plot

Name: Prednisone; Processing Method: Prednisone Proc Mth; Fit Type: Linear (1st Order); Cal Curve Id: 2973; A: 0.000000e+000; B: 4.867524e+007; C: 0.000000e+000; D: 0.000000e+000; F/2: 1.00000
Quantitative work in HPLC

Quantitative Results

**Sample Information**

- **Sample Name:** K-29825-1-H2O2
- **Sample Type:** Unknown
- **Vial:** 24
- **Injection #:** 1
- **Injection Volume:** 20.00 µl
- **Run Time:** 50.0 Minutes

**Acquired By:** Sofia

**Date Acquired:** 14-09-2000 01:22:17 IDT

**Date Processed:** 17-04-2010 07:56:42 IDT

---

**PDA Report: Example**

![PDA Spectrum Index Plot](image)

**PDA Result Table**

<table>
<thead>
<tr>
<th>Name</th>
<th>RT</th>
<th>Purity1</th>
<th>Purity1 Threshold</th>
<th>Purity1 Flag</th>
<th>Match1</th>
<th>Spec. Name</th>
<th>PDA/FLR Match1</th>
<th>Match1 Flag</th>
<th>PDA/FLR Match1 Flag</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.737</td>
<td>1.854</td>
<td>2.483</td>
<td>No</td>
<td>Main 100%</td>
<td>Training_Nov09</td>
<td>1.732</td>
<td>2.489</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>8.611</td>
<td>0.261</td>
<td>0.439</td>
<td>No</td>
<td>Main 100%</td>
<td>Training_Nov09</td>
<td>4.183</td>
<td>1.142</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>11.663</td>
<td>0.041</td>
<td>0.240</td>
<td>No</td>
<td>Main 100%</td>
<td>Training_Nov09</td>
<td>0.824</td>
<td>1.068</td>
<td>No</td>
</tr>
</tbody>
</table>

---

**% Difference between Standards**

\[
\text{Std A} = 0.0034965 - \text{Std B} = 0.0047810 \\
\text{WA / \text{Std A} / \text{Std B}} = 0.0047810 - 0.0034965
\]

**Area_Solu_Factor**

\[
\text{Solu} = 0.0034965 \\
\text{Solu / Solu - 0.0047810} = 0.0034965
\]
Quantitative work in HPLC

**Match Plot**

Sample Name: K-26525/1-H2O2 Vial: 24 Injection: 1 Date Acquired: 14/09/2000 01:22:17 IDT Name: Deg1 Match1 Angle: 4.183 Match1 Threshold: 1.142 Match1 Spect. Name: Main 100%

**Match Plot**

Sample Name: K-26525/1-H2O2 Vial: 24 Injection: 1 Date Acquired: 14/09/2000 01:22:17 IDT Name: Deg1 Match1 Angle: 0.024 Match1 Threshold: 1.008 Match1 Spect. Name: Main 100%

**Purity Plot**

Sample Name: K-26525/1-H2O2 Vial: 24 Injection: 1 Date Acquired: 14/09/2000 01:22:17 IDT Purity1 Angle: 0.261 Purity1 Threshold: 0.439

**Purity Plot**

Sample Name: K-26525/1-H2O2 Vial: 24 Injection: 1 Date Acquired: 14/09/2000 01:22:17 IDT Purity1 Angle: 0.041 Purity1 Threshold: 0.240

**Reports to Connect**

Data to Decisions
Quantitative work in HPLC

Electronic Signatures

The Manual Process

LIMS

Chromatographic Data System (CDS)

Additional Calculations Performed

Reports Compiled

Final Review

Signatures Applied

Reports Reviewed

94
Quantitative work in HPLC

The Manual Process
Chromatography to Calculations

Empower CDS

Additional Calculations Performed

The Manual Process
Review and Signature

Reports Compiled
Reports Reviewed
Signatures Applied
Final Review

95
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Quantitative work in HPLC

Electronic Review and Signature Process