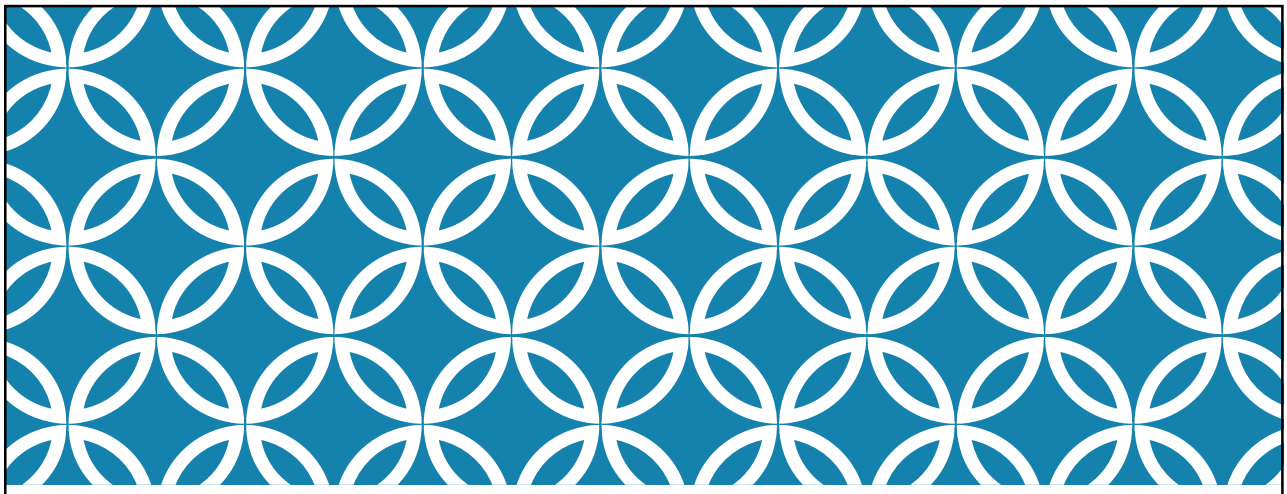

HPLC METHOD VALIDATION BASICS

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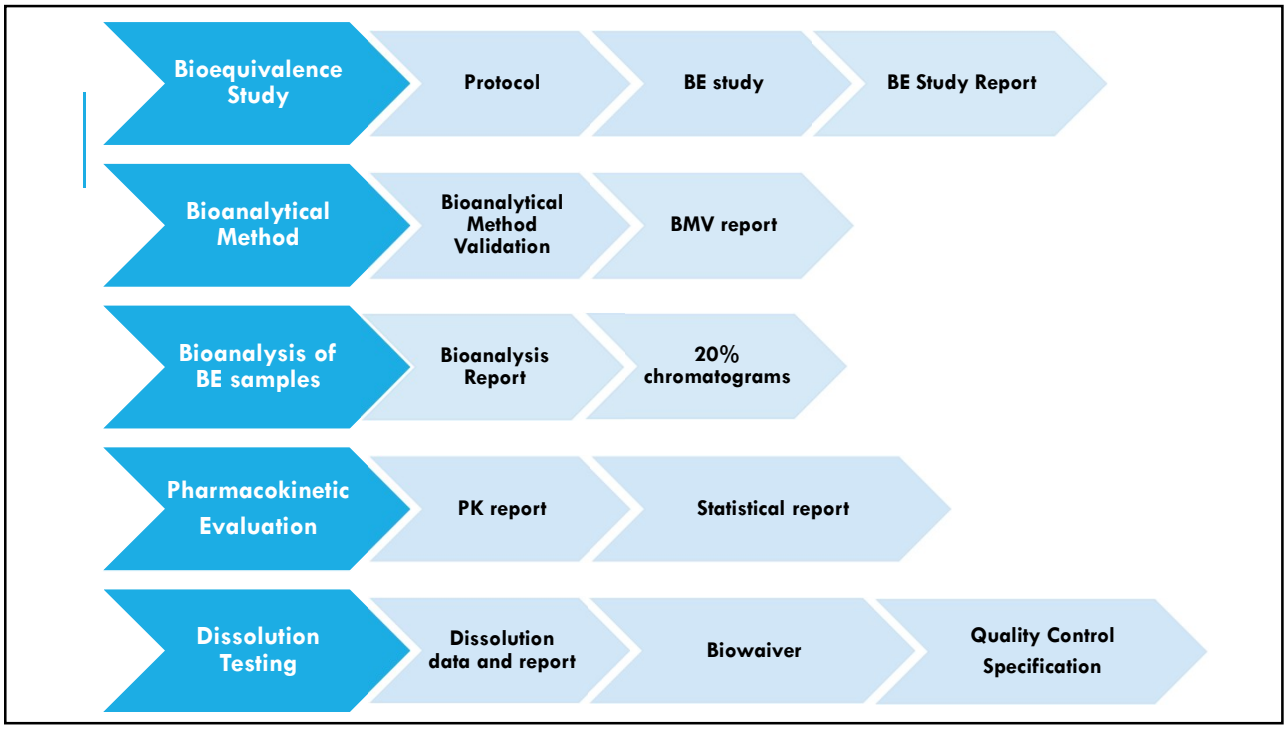
Bioanalytical Webinar Training

DISCLAIMER

- The views and opinions presented here represent those of the speaker and should not be considered to represent advice or guidance on behalf of the U.S. Food and Drug Administration or the Saudi Food and Drug Authority



OUTLINE – NEXT PRESENTATIONS

- **Presentation 2**
- HPLC methodology
- Content of HPLC test procedure
- System Suitability Testing (SST)
- Validation of HPLC analytical procedure (validation parameters role).
  - Selectivity
  - Sensitivity (LLOQ)
  - Linearity
  - Accuracy and Precision
  - Ruggedness

- **Presentation 3**
- Small Molecule Quantitation
- Method Development
- Method Validation
  - Definitions
- Method Validation criteria

OUTLINE

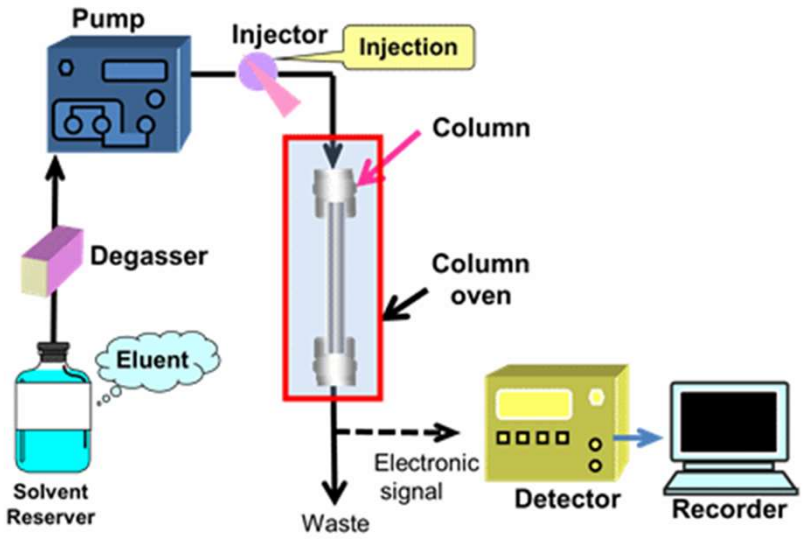
- HPLC methodology
- Content of HPLC test procedure
- System Suitability Testing (SST)
- Validation of HPLC analytical procedure (validation parameters role).
  - Selectivity
  - Sensitivity (LLOQ)
  - Linearity
  - Accuracy and Precision
  - Ruggedness

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

- HPLC is a separation technique based on a solid stationary phase and a liquid mobile phase. Separations are achieved by partition, adsorption, or ion-exchange processes, depending upon the type of stationary phase used.
  - Chiral:
  - Ion-exchange:
  - Ion--pair/affinity:
  - Normal phase:
  - Reversed phase:
  - Size exclusion:
- The reversed-phase HPLC with UV detection is most commonly used form of HPLC, is selected to illustrate the parameters of HPLC method and validation.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

- **Chiral:** Separation of the enantiomers can be achieved on chiral stationary phases by formation of diastereomers via derivatizing agents or mobile phase additives on achiral stationary phases.
- **Ion-exchange:** Separation is based on the charge-bearing functional groups, anion exchange for sample negative ion (XI, or cation exchange for sample positive ion (X^+).
- **Ion--pair/affinity:** Separation is based on a chemical interaction specific to the target species.
- **Normal phase:** Normal phase chromatography is a chromatographic technique that uses organic solvents for the mobile phase and a polar stationary phase.
- **Reversed phase:** Separation based on solvent strength and selectivity also may be affected by column temperature and pH. In general, the more polar components elute faster than the less polar components.
- **Size exclusion:** separation is based on the molecular size or hydrodynamic volume of the components.



CONTENT OF HPLC TEST PROCEDURE

- Any analytical procedure submitted should be described in sufficient detail, includes:
  - Preparation of mobile phase
  - Chromatographic condition:
    - Column: type (e.g., C18 or C8), dimension (length, inner diameter), particle size (10µm, 5 µm)
  - Detector: wavelength
  - Injection volume
  - Flow rate

CONTENT OF HPLC TEST PROCEDURE

- Elution procedure:
  - isocratic or gradient elution
- Preparation of standards and samples
- Operation procedure: sequence of injections
- System suitability testing (**SST**) and Validation criteria
- Calculations

COMPENDIAL METHODS

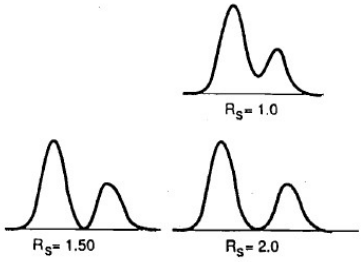
- When claim a compendial method, there should be no change in:
  - The type of column i.e the stationary phases
  - Detector wavelength
  - Components in Mobile phase
  - System suitability testing and criteria
- Adjustments to ratio of components in mobile phase, flow rate, column temp, dimension of column, particle size (reduction only), may be necessary to achieve the system suitability criteria.
- The allowable variations for each parameter, see USP general chapter <621>.

SYSTEM SUITABILITY TESTING (SST)

- **Setting System Suitability Specifications:**
- Tailing Factor < 2.5 (allows for higher sample load)
- Resolution > 2.0 (allows for method variation and column aging)
- RSD of replicate injections < 2.0% (checks system performance)

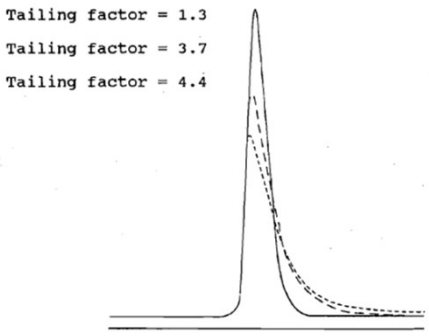
SYSTEM SUITABILITY TESTING (SST)

- Precision:
  - Assay: RSD ≤1% (API) or ≤ 2% (FPP), n ≥ 5
  - Impurities: in general, RSD ≤ 5% at the limit level, up to 10% or higher at LOQ, n ≥ 6
- Resolution (R): >2



SYSTEM SUITABILITY TESTING (SST)

- Tailing factor/peak asymmetry: (≤ 2)

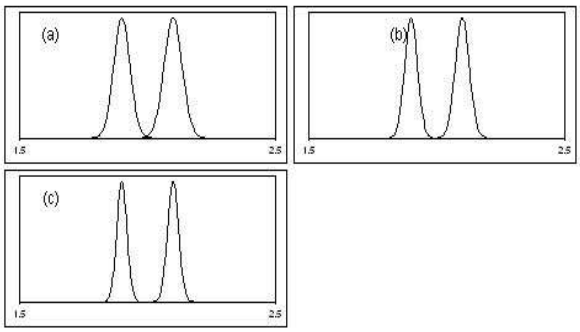


SYSTEM SUITABILITY TESTING (SST)

- Number of theoretical plates (N): column efficiency ≥ 2000

- Gradient elution is one way to increase the N

Theoretical models of peaks obtained on columns packed with three different particle sizes: retention times 1.9 & 2.1 min on a 50mm column (a) particle size, d_p=5µm; theoretical plates, N=3500; resolution, R=1.48 (b) particle size, d_p=3µm; theoretical plates, N=5800; resolution, R=1.90 (c) particle size, d_p=1.9µm; theoretical plates, N=9200; resolution, R=2.40



SYSTEM SUITABILITY TESTING (SST)

- A SST should contain:
  - For Assay:
    - precision + one or more other parameter
  - For impurity test:
    - resolution + precision + one or more other parameter

WHAT IS "VALIDATION OF ANALYTICAL METHODS"?

- Scientifically demonstrating that the analytical methods concur with the intended purpose (i.e., that errors are within a permissible range)

Validation characteristics

- Accuracy / trueness
- Precision
- Specificity
- Detection limit
- Quantitation limit
- Linearity
- Range
- (Robustness)

ICH, USP, and FDA Methods Validation Characteristics Requirements for Various Types of Tests

Validation Characteristics	Assay	Testing for Impurities		Identification
		Quantitative	Limit	
Accuracy	Yes	Yes	No	No
Precision - Repeatability	Yes	Yes	No	No
Precision - Intermediate Precision	Yes[1]	Yes*	No	No
Specificity	Yes	Yes	Yes	Yes
Detection limit	No	No	Yes	No
Quantitation limit	No	Yes	No	No
Linearity	Yes	Yes	No	No
Range	Yes	Yes	No	No
Robustness	Yes	Yes	No	No

* In cases where reproducibility has been performed, intermediate precision is not needed.[7]

USP Data Requirements for Method Validation

Parameter	Bulk Drug	Impurities Degradates	Product Performance
Precision	Yes	Yes	Yes
Accuracy	Yes	Yes	Maybe
Limit of Detection	No	No	Maybe
Limit of Quantitation	No	Yes	Maybe
Specificity/Selectivity	Yes	Yes	Maybe
Range	Yes	Yes	Maybe
Linearity	Yes	Yes	Maybe
Ruggedness	Yes	Yes	Yes

VALIDATION – COMPENDIAL METHODS

Assay – API

- No validation generally required. Exception: specificity for major impurities not in the monograph.

Assay – Finished Pharmaceutical Product (FPP

- Specificity, accuracy and precision (repeatability).

Purity – API and FPP

- Full validation for specified impurities that are not included in the monograph (specificity, linearity, accuracy, repeatability, intermediate precision, LOD/LOQ)
- Validation of the limit for individual unknowns, if tighter than that in the monograph: LOQ of the API should be below the limit for individual unknowns

NON-COMPENDIAL METHODS

- Full validation is required for purity, assay and dissolution methods (HPLC, UV) :
  - Specificity
  - Linearity
  - Accuracy
  - Repeatability
  - Intermediate precision
  - LOD/LOQ (not required for assay, dissolution)
  - Robustness (recommended)

SPECIFICITY

Definition

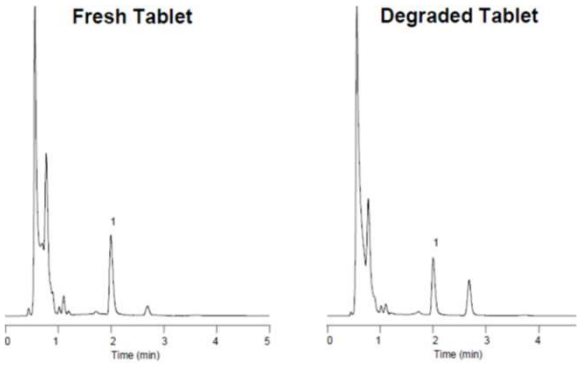
- The ability to accurately analyze the target substance in the presence of other expected substances
- The discrimination capability of the analytical methods
- Multiple analytical procedures may be combined in order to attain the required level of discrimination

Evaluation Method

- Confirmation that the target substance can be discriminated (separated) from co-existing components, related substances, decomposition products, etc.
- If reference standards for impurities cannot be obtained, the measurement results for samples thought to contain the impurities are compared.

SPECIFICITY

- Blank solution to show no interference
- Placebo to demonstrate the lack of interference from excipients
- Spiked samples to show that all known related substances are resolved from each other
- Stressed sample of about 10 to 20% degradation is used to demonstrate the resolution among degradation products
- Representative chromatograms should be provided with time scale and attenuation indicated



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LINEARITY/RANGE

- Definition - Range
  - The region between the lower and upper limits of the quantity of a target substance that gives appropriate levels of accuracy and precision
- Definition - Linearity
  - The ability of the analytical method to produce measurements for the quantity of a target substance that satisfy a linear relationship.
  - Values produced by converting quantities or measurements of the target substance using a precisely defined formula may be used.
- Evaluation Method - Range
  - The accuracy, precision, and linearity are investigated for samples containing quantities of a target substance that correspond to the lower limit, upper limit, and approximate center of the range.
- Evaluation Method - Linearity
  - Samples containing different quantities of the target substance (usually 5 concentrations) are analyzed repeatedly, and regression equations and correlation coefficients are obtained.
  - Residuals obtained from the regression equations of the measurements are plotted, and it is confirmed that there is no specific slope

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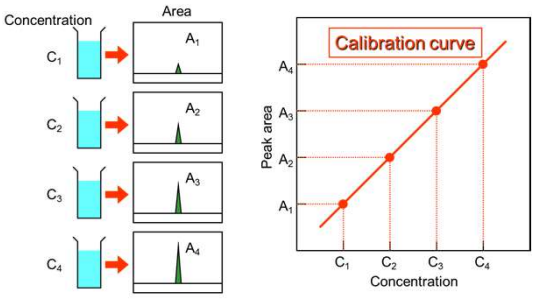
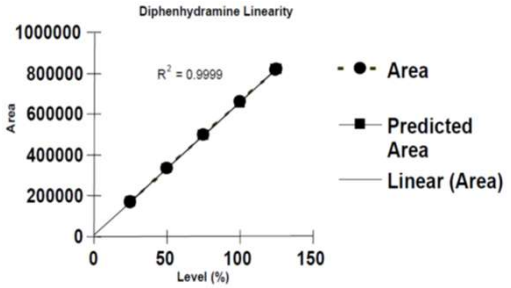
LINEARITY / RANGE

- The working sample concentration and samples tested for accuracy should be in the linear range (concentrations Vs. Peak areas)
- **Minimum 5 concentrations**
- Dilute of stock solution or separate weighing's
- must cover a range of 80 - 120% of the expected concentration

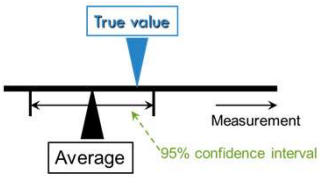




LINEARITY / RANGE

- Correlation coefficient (r)
  - API: ≥ 0.998
  - Impurities: ≥ 0.99
- y-Intercept and slope should be indicated together with plot of the data

ACCURACY / TRUENESS



- Definition
- Describes the closeness of the mean test results obtained by the method to the actual concentration value of the analyte (nominal concentration). Measured as % accuracy or % bias and should be ± 15% (± 20% for LLOQ).
- Degree of bias in measurements obtained with analytical procedures
- Difference between true value and grand mean of measurements

- Evaluation Method
  - Comparison with theoretical values (or authenticated values)
  - Comparison with results obtained using other analytical procedures for which the accuracy (trueness) is known
  - Recovery test

$$\%\,Accuracy = \frac{Actual - Measured}{Actual} \times 100$$

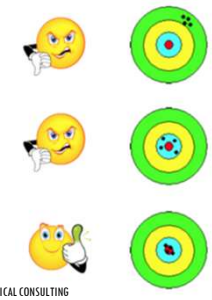
$$\%\,Bias = \left[1 - \frac{Measured}{Actual}\right] \times 100$$

ACCURACY

- **Assay**
  - **API**: against an RS of known purity, or via an alternate method of known accuracy; analysis in triplicate.
  - **FPP**: samples/placeboes spiked with API, across the range of **80 - 120% of the target concentration, 3 concentrations**, in triplicate each.
- Report percent recovery (mean result and RSD): 100 ± 2%
- ICH Q2 states: accuracy may be inferred once precision, linearity and specificity have been established.
- **Impurities**: API/FPP spiked with known impurities

ACCURACY

- **Impurities:** API/FPP spiked with known impurities
- Experienced in PQ:
- Across the range of **LOQ-150%** of the target concentration (shelf life limit), 3-5 concentrations, in triplicate each. (LOQ, 50%, 100%, 150%)
- **Per cent recovery:** in general, **within 80-120%**, depends on the level of limit



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PRECISION

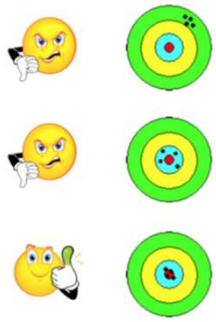
- Definition
  - Degree of concurrence of series of measurements obtained by repeatedly analyzing multiple samples taken from a homogenous test substance
  - Variance, standard deviation, or relative standard deviation of measurements
- Repeatability / Intra-Assay Precision
  - Precision of measurements taken over a short time period under the same conditions
- Intermediate Precision
- Reproducibility

$$\%CV = \frac{S.D.}{Mean} \times 100$$

LCK PHARMACEUTICAL CONSULTING

PRECISION

- **System precision:**
  - by multiple injections (n ≥5) of a homogeneous sample (standard solution).
  - RSD ≤ 1% is recommended for assay;
  - RSD ≤ 5% is recommended for related substances (reference standards at the limit)
  - Indicates the performance of the HPLC system
  - As a system suitability test



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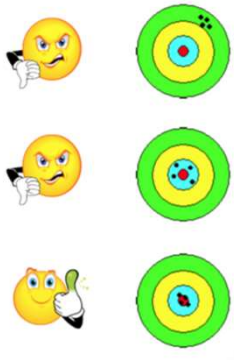
PRECISION

- Repeatability (method precision)
  - Multiple measurements of a sample by the same analyst
  - A minimum of 6 determinations at the test concentration (6 times of a single batch), or
  - 3 levels (80%, 100%, 120%) , 3 repetitions each (combined with accuracy)
  - For Assay: RSD ≤ 2.0%
  - For individual impurity above 0.05%, in general, RSD ≤ 10%



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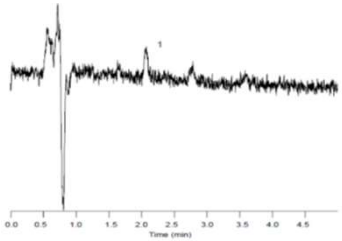
PRECISION

- Intermediate precision (part of ruggedness)
  - Test a sample on multiple days, analysts, instruments
  - Repeat the method precision by different analyst in different equipment using different lot of column on different days
  - RSD should be the same requirement as method precision
- Reproducibility (inter-laboratory trial)
  - Not requested in the submission

DETECTION LIMIT

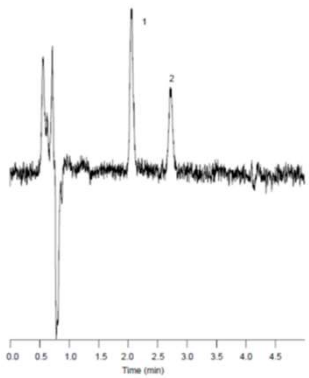
- **Definition**
  - The minimum quantity of a target substance that can be detected.
  - Quantitation is not absolutely necessary.



- **Evaluation Method**
  - Calculated from the standard deviation of measurements and the slope of the calibration curve.
    - $DL = 3.3\ \sigma/slope$
      (σ: Standard deviation of measurements)
      (Slope: Slope of calibration curve)
  - Calculated from the signal-to-noise ratio.
    - Concentration for which S/N = 3 or 2

QUANTITATION LIMIT

- Definition
  - The minimum quantity of a target substance that can be quantified
  - Quantitation with an appropriate level of accuracy and precision must be possible. (In general, the relative standard deviation must not exceed 10%.)



- Evaluation Method
  - Calculated from the standard deviation of measurements and the slope of the calibration curve.
    - $QL = 10\ \sigma/slope$
      (σ: Standard deviation of measurements)
      (Slope: Slope of calibration curve)
  - Calculated from the signal-to-noise ratio.
    - Concentration for which S/N = 10

LOD/LOQ

- Should be validated by analysis of samples at the limits.
  - **LOD: below the reporting threshold**
  - **LOQ: at or below the specified limit**
- Not required for assay/dissolution methods.
- Applicant should provide
  - the method of determination
  - the limits
  - chromatograms

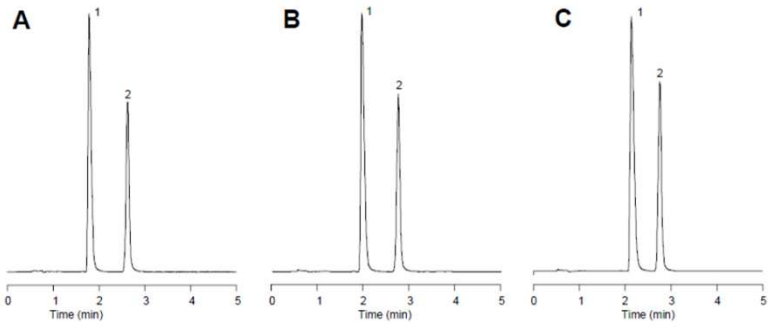
ROBUSTNESS

- The method's capability to remain unaffected by small but deliberate variations in method parameters
  - Influence of variations of pH in a mobile phase
  - Influence of variations in mobile phase composition
  - Different columns (different lots and/or suppliers)
  - Temperature
  - Flow rate
- Evaluate the System suitability parameters
- Some or all of the variable factors (i.e., the analytical conditions) are changed and the effects are evaluated.

PH VARIATION

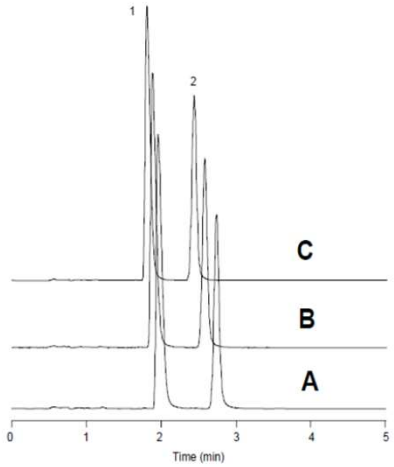
- Tested pH at 4.0, 4.5, and 5.0.
- Monitor for substantial changes in retention, resolution, and peak shape

	pH	Time	R_s	T_f
A	4.0	1.78	6.2	1.7
B	4.5	1.94	5.5	1.7
C	5.0	2.14	4.0	1.8



Sample: 1. Diphenhydramine 0.5 mg/mL 2. Benzophenone .005 mg/mL

TEMPERATURE

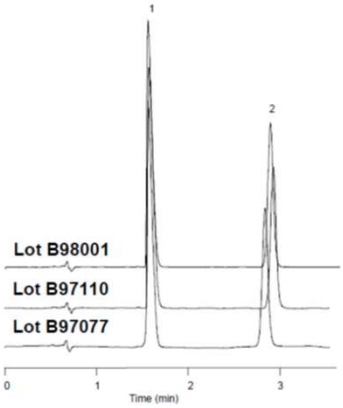


	°C	α	T_f
A	RT	1.6	1.8
B	30	1.6	1.7
C	35	1.6	1.7

- Tested 3 temperatures – Room Temperature, 30°C and 35°C
- Monitor for changes in selectivity (α) and peak shape of diphenhydramine

Sample: 1. Diphenhydramine 0.5 mg/mL 2. Benzophenone .005 mg/mL

COLUMN LOT



- Compare three current lots of material for consistency of retention (k) and selectivity (α).

Three Lot Summary

	Mean	SD	RSD
k (D)	1.1	0.01	1.0%
k (B)	2.9	0.06	2.1%
α	2.6	0.05	1.8%

Sample: 1. Diphenhydramine 0.5 mg/mL 2. Benzophenone .005 mg/mL

RUGGEDNESS/REPRODUCIBILITY

- Multiple chemists in multiple labs run samples.
- Results should be reproducible and can be compared to method precision.
- Samples were run in 3 labs by 3 chemists on 3 different instruments.

Level	Chemist 1 Accuracy/RSD	Chemist 2 Accuracy/RSD	Chemist 3 Accuracy/RSD
125%	99.6 +/- 0.2%	100.2 +/- 0.8%	99.0 +/- 0.8%
75%	100.3 +/- 0.8%	100.5 +/- 0.0%	100.5 +/- 0.3%
125%	99.2 +/- 0.7%	100.6 +/- 0.0%	101.0 +/- 0.7%
Overall	99.7 +/- 0.9%	100.4 +/- 0.4%	100.2 +/- 1.0%
Method	100.0 +/- 0.9%		

CONCLUSION

- HPLC methods play a critical role in analysis of pharmaceutical product
- Validation of HPLC should demonstrate that the method is suitable for its intended use
- Data for acceptance, release, stability will only be trustworthy if the methods used are reliable

QUESTIONS

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