Validation of an analytical method on a HPLC/UPLC-spectrophotometry endpoint

A. Background

The approach used to validate an analytical method on a HPLC/UPLC-spectrophotometry system is based on The Federal Drug Administration (FDA) guidance for industry from May 2001 on Bio-analytical Method Validation (US FDA, 2001). Within the FDA guidance, validation of a bio-analytical method encompasses all of the procedures that demonstrate a particular bio-analytical method used for quantitative measurement of analytes in a given biological matrix is reliable and reproducible for intended use. The fundamental parameters for such a validation include: 1) selectivity; 2) precision and accuracy; 3) matrix effect; 4) carryover; 5) reproducibility and 6) stability. Validation involves documenting, through the use of specific laboratory investigations that the performance characteristics of the method are suitable and reliable for the intended analytical applications. The acceptability of analytical data corresponds directly to the criteria used to validate the system detection method.

B. Validation of an analytical method on a HPLC/UPLC-spectrophotometry system

Five different sample types were prepared to enable evaluation of key parameters for the approach to qualify the HPLC/UPLC endpoint. These were as follows:

- Solvent: isopropanol (IP)
- Living Blank: IP extract of a living tissue without MTT. This sample is an untreated SkinEthic™ RHE tissue on which the IP extraction step only is conducted. The same pool of blanks sample is used for the period of the qualification.
- Dead Blank: IP extract of killed tissues without MTT: The killed tissues are obtained following the step describes in section B.1.4.
- Standard sample: sample in IP with known concentration of formazan (CAS number 57360-69-7; purity > 97% or equivalent) is used to prepare the calibration curves. In this context, two limits are defined as follows:
    - Upper Limit Of Quantification (ULOQ) defined as being at least twice as high as untreated sample expressed as formazan concentration (i.e. for 200% cell viability).
    - Lower Limit Of Quantification (LLOQ) defined to enable the calibration curve to cover two orders of magnitude (i.e. 2% cell viability).

  Six concentrations are chosen from 0.823 to 200 µg/mL (1/3 dilutions) which cover the 2% cell viability at the lowest end of the concentration range and at least two times the highest Reconstructed human Tissue concentration for the upper part of the concentration range.
- QC samples: Sample in IP with known concentration of formazan at three different levels: low, medium and high. Concentrations of the QC samples are chosen to be with a constant factor between them and identified as:
    - QC low: 2 x LLOQ (i.e. 1.6 µg/mL)
    - QC medium: 16 µg/mL
    - QC high: 0.8 x ULOQ (i.e. 160 µg/mL)

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C. Samples preparation

The different samples were prepared as follows:

- Stock solution :
  - Weigh 10 mg of formazan in an appropriate glass container and add 10 mL of IP (1000 µg/mL)
  - Stir overnight at room temperature with a magnetic bar (store up to 6 months in the freezer)
- ULOQ preparation:
  - Prepare a dilution of a factor 5 from the stock solution in IP using an appropriate container (ULOQ = 200 µg/mL) : 300 µL of stock solution + 1200 µL IP
- Calibration curve samples:
  - From ULOQ: 6 serial 1/3 dilutions in IP to obtain the following concentrations:
    i. 200 µg/mL
    ii. 66.6 µg/mL (1000 µL IP + 500 µL solution at 200 µg/mL)
    iii. 22.2 µg/mL (1000 µL IP + 500 µL solution at 66.6 µg/mL)
    iv. 7.41 µg/mL (1000 µL IP + 500 µL solution at 22.2 µg/mL)
    v. 2.47 µg/mL (1000µL IP + 500 µL solution at 7.41 µg/mL)
    vi. 0.823 µg/mL (1000 µL IP + 500 µL solution at 2.47 µg/mL)
- QC samples preparation:
  - QC stock solution (QC stock solution): 1600 µg/mL in IP: weigh 16 mg of formazan in an appropriate glass container and add 10 mL of IP
  - QC high (160 µg/mL): 1/10 dilution of the QC stock solution in IP or living blank
  - QC medium (16 µg/mL): 1/10 dilution of the QC high in IP or living blank
  - QC low (1.6 µg/mL): 1/10 dilution of the QC medium in IP or living blank

D. HPLC/UPLC-spectrophotometry analytical conditions

- Each laboratory established specific conditions of operation for their HPLC/UPLC-spectrophotometry system. For example the L'Oréal system is an HPLC with UV-Visible detection. Use of a photodiode array detector was preferred rather than a single wavelength detector so that the quantified formazan could be confirmed by its spectrum identity. The chromatographic system used was an HPLC Waters Alliance 2695 with a Waters UV PAD 2996 detector, controlled with Empower Pro v.5.00 with analytical conditions as follows:

  - Column: Waters Xterra RP18 5µm 150*4.6mm
  - Column temperature: 50°C
  - Mobile phases A and B: HPLC grade Water and HPLC grade Acetonitrile
  - Flow rate: 1mL/min
  - Injection volume: 10 µL
  - Needle wash: IP
  - Seal wash in: water/methanol (90/10 v/v)
  - Stroke volume (µL): automatic
  - Syringe draw rate (µL/sec): slow

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- Needle wash time: extended
- Wave length range: 250-700 nm
- Sampling rate: 2.0 point / sec
- Resolution: 1.2 nm
- Gradient mode during 10 minutes was as detailed in the following table:

Time (Min)	Solvent A	Solvent B
0	100	0
1	100	0
3	0	100
6	0	100
6.1	100	0
10	100	0

Before the first injection the system was equilibrated during 20 minutes in the initial conditions (100% solvent A).

- HPLC reading internal control: during an analytical sequence, IP (blank matrix) is injected once and QC samples at 20 µg/mL and 200 µg/mL of formazan is injected approximately every 20 injections. After the QC samples another IP injection is done.

Data reading and storage

- Measure peak area of the formazan for each sample
- All data generated by the HPLC/UPLC-spectrophotometry should be printed (or saved as a '.pdf' files) and considered as raw data.
- Link Area values with the appropriate treatment conditions on the raw data documents (or files).
- Perform the Quality Control of the raw data.

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E. Acceptance criteria for the **validation of an analytical method on a HPLC/UPLC-spectrophotometry system**

E.1. Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte - here formazan - in the presence of other components in the sample.

For selectivity, analysis of blank samples of the appropriate biological matrix (here IP tissue extract) are obtained from at least 6 sources (i.e. at least 3 killed tissues - dead blank and 3 living tissues - living blank) from the same batch of tissues.

Optional: Dye (methylene blue CAS number 7220-79-3; maximum wavelength ~650 nm obtained from Sigma) is added to demonstrate that the system could quantify formazan in the presence of a color test chemical. In this way, IP containing methylene blue dye is evaluated. Nevertheless, it doesn't demonstrate absolute selectivity of the analytical method. It has to be controlled for each tested chemical.

Each blank sample is tested for interference with selectivity needing to be assured at the Lower Limit of Quantification (LLOQ).

E.2. Precision and Accuracy

Precision of an analytical method describes the closeness of individual measures of an analyte - here formazan - when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of matrix. The precision is expressed by the Coefficient of Variation (CV) calculated using the following relationship:

$$\text{CV } \% = \frac{\text{Standard Deviation}}{\text{Mean}} \text{ x } 100$$

Precision is further subdivided into 1) intra-batch precision and repeatability in a single analytical run, and 2) the inter-batch precision and repeatability between runs.

The accuracy of the analytical method will describe the closeness of mean test results obtained by the method to the true value (concentration) of the analyte - here formazan. Accuracy was determined by replicate analysis of samples containing known amounts of formazan using the following relationship:

$$\% \text{ Accuracy} = \frac{(\text{Concentration}_{\text{Mean measured}} - \text{Concentration}_{\text{True}})}{\text{Concentration}_{\text{True}}} \text{ x } 100$$

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Drawing from the FDA Guideline, precision and accuracy in IP were measured at 3 different QC formazan concentrations over 3 consecutive days on independent samples and stock solutions. Five replicates of QC samples are quantified on the first day over the three calibration curves performed. On the 2 following days, 3 replicates of the QC samples are quantified over the calibration curve performed the same day. The acceptance criterion was established that the precision determined at each QC level was not to exceed 15 % of CV for each independent determination. Furthermore, the mean determination values was expected to be within 15 % of the true value for each of the three QC levels over the calibration curves performed the same day.

E.3. Matrix Effect (here identified as RhT tissue insert effect)

Reconstructed human Tissues (RhT) insert effect is identified as the Matrix Effect (ME) that reflects the extraction efficiency of an analytical method within the limits of variability. The Matrix Effect (Rht tissue insert effect) is calculated using the following relationship:

$$\mathrm{ME}\ \% = \frac{\mathrm{Concentration}_{\mathrm{living\ blank}}}{\mathrm{Concentration}_{\mathrm{IP\ solution}}} * 100$$

The matrix here is defined as the IP extract from the living blank. The Matrix Effect of the formazan in an assay is the detector response obtained from an amount of formazan added to living blank, compared to the detector response obtained for the true concentration of the pure authentic formazan standard (in IP).

Examination of the Matrix Effect is necessary to ensure that the difference between the standard sample (i.e. IP solution) and unknown samples (*i.e.* living blank) does not affect the true formazan concentration measured.

Matrix Effect (RhT tissue insert effect) for the determination of formazan was measured on one day using 5 independent samples of the same stock solution at the three QC concentrations (living blank samples compared to the calibration curve). The acceptance criterion for Matrix Effect (RhT tissue insert effect) is established that the mean value of the 5 samples at each QC concentration be between 85 % and 115 % according to the above relationship. Percentage of CV is measured for the three QC concentrations and meant not to exceed 15 %.

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E.4. Carryover (Cross-contamination)

Carryover (cross-contamination) corresponds to the amount of residual formazan in the analytical system after analysis of the ULOQ sample. Carryover should be as low as possible to avoid over-estimation of unknown formazan samples.

To measure the carryover, a solvent sample is analyzed after the run of an ULOQ standard. The acceptance criterion is established that the area of the carryover is meant not to exceed 20 % of the LLOQ area.

E.5. Calibration/Standard Curve and Robustness/Reproducibility

A calibration (standard) curve demonstrates the relationship between instrument response area and known concentrations of the formazan. A sufficient number of standards need be used to adequately define the relationship between concentration and response. The calibration curve covers a given range of concentrations defined by the LLOQ and ULOQ.

The calibration curve for formazan is generated with at least 6 standard samples that included the LLOQ and ULOQ. A linear relation (forced through zero) between concentration and area is established. Such simple linear relation y = x axis, allows simplifying the further analytical process using a single point calibration. The concentrations of formazan used to generate the standard calibration curve are provided in the following table.

	Formazan concentrations (µg/mL) (CAS # 57360-68-7, Sigma or equivalent)
Calibration curve linear through zero (y = x axis)	0.823, 2.47, 7,41, 22.2, 66.6, 200

The following conditions are to be met in preparing the calibration curve:

Maximum 20 % deviation of the LLOQ from true concentration

Maximum 15 % deviation of standards other than LLOQ from true concentration

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The deviation is calculated as follows:

$$\%\ \text{Dev} = \frac{(\text{Concentration}_{\text{measured}} - \text{Concentration}_{\text{True}})}{\text{Concentration}_{\text{True}}}\ \text{x}\ 100$$

Robustness corresponds to the ability of the analytical method to give similar results for the calibration curve within a day and from day to day. It is assessed with precision measurements.

Robustness which allows evaluation of the robustness and reproducibility of the quantification method intra- and inter-days is addressed as follows:

Intra-day: the calibration curve is repeated three times the same day with three different stock solutions. The QC samples series prepared with a fourth independent solution allowed measurement of within-run precision and accuracy with the three calibration curves.

Inter-day: A calibration curve is repeated on three consecutive days with three different stock solutions. Each day, a QC samples series prepared with an independent solution allowed measurement of precision and accuracy from day to day.

E.6. Stability

Formazan stability is a function of storage conditions. The stability of formazan in a particular matrix (i.e. IP, living blank) and containers (e.g. well plate, HPLC vial) is relevant only to the matrix and container used and is not extrapolated to other matrices and containers.

Stability procedures evaluated the stability of the formazan during sample collection and handling, short-term (bench top, room temperature) storage and after long-term (at the intended temperature) storage. Conditions used in stability experiments reflected situations likely to be encountered during actual sample handling and analysis.

Evaluation of stability also included an evaluation of formazan stability in stock solution. Such stability evaluation enabled use of the formazan stock solution over the period of the validation of the analytical method on HPLC/UPLC-spectrophotometry.

All stability determinations used a set of samples prepared from a freshly made stock solution of the formazan in the appropriate formazan-free IP solution and living blank. Stock solutions of the formazan for stability evaluation are prepared in an appropriate solvent (i.e. IP) at known concentration.

Formazan stability is evaluated at the three QC concentrations in three independent replicates in solvent as well as in living tissues blank. Stability is tested by comparing the area with those of freshly prepared samples. The accuracy of a stability sample is identified as meant to be within 15 % of the fresh sample.

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Four different stability scenarios are evaluated. These are:

Stock solution stability: The stability of stock solutions of formazan is evaluated at room temperature for at least 6 hours.

Auto-sampler stability (doped solvent and doped living blank): The stability of the formazan is assessed over the anticipated run time for the batch size. Samples are kept on the auto-sampler until the next measurements. Evaluation of re-injections from the same vials is included.

Short term lab stability (doped living blank): This corresponded to the time frame that is to be expected in performance of the *in vitro* skin irritation test method i.e. completion of test method procedures including tissue treatment, spectrophotometry reading and data interpretation before the samples would be analyzed by HPLC/UPLC-spectrophotometry from 4 to 24 hours (based on the expected duration that samples would remain at room temperature in the intended study).

Long term storage stability (doped living blank): Evaluation of long-term stability enabled unknown samples to be analyzed up to at least a month at -20°C after generation. In this circumstance, clear definition of storage conditions is required (i.e. in the refrigerator or at room temperature according to the laboratory storage conditions).

Using the approach in the US FDA guidance document as a basis application of these key parameters, with associated acceptance criteria, to the validation of the analytical method on HPLC/UPLC-spectrophotometry for measurement of formazan to demonstrate acceptability of this analytical technique as an additional endpoint is summarized in the Table below.

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Key parameters and acceptance criteria for qualification of an HPLC/UPLC-photometry system for measurement of MTT formazan extracted

Parameter	Protocol Derived from FDA Guidance	Acceptance Criteria
Selectivity	Analysis of isopropanol, living blank (isopropanol extract from living tissues without any treatment), dead blank (isopropanol extract from killed tissues without any treatment), and of a dye (e.g., methylene blue)	$Area_{interference}$ ≤ 20% of $Area_{LLOQ}$[1]
Precision	Quality Controls (MTT formazan) in isopropanol (n=5)	CV ≤ 15% or ≤ 20% for the LLOQ
Accuracy	Quality Controls in isopropanol (n=5)	%Dev ≤ 15% or ≤ 20% for LLOQ
Matrix Effect	Quality Controls in living blank (n=5)	85% ≤ ME% ≤ 115%
Carryover	Analysis of isopropanol after an ULOQ[2] standard	$Area_{interference}$ ≤ 20% of $Area_{LLOQ}$
Reproducibility (intra-day)	3 independent calibration curves (based on 6 consecutive 1/3 dilutions of MTT formazan in isopropanol starting at ULOQ); Quality Controls in isopropanol (n=5)	Calibration Curves: %Dev ≤ 15% or ≤ 20% for LLOQ Quality Controls: %Dev ≤ 15% and CV ≤ 15%
Reproducibility (inter-day)	Day 1: 1 calibration curve and Quality Controls in isopropanol (n=3) Day 2: 1 calibration curve and Quality Controls in isopropanol (n=3) Day 3: 1 calibration curve and Quality Controls in isopropanol (n=3)	
Short Term Stability of MTT Formazan in Tissue Extract	Quality Controls in living blank (n=3) analysed the day of the preparation and after 24 hours of storage at room temperature	%Dev ≤ 15%
Long Term Stability of MTT Formazan in Tissue Extract, if required	Quality Controls in living blank (n=3) analysed the day of the preparation and after several days of storage at -20°C	%Dev ≤ 15%

[1]LLOQ: Lower Limit of Quantification, defined to cover 1-2% tissue viability
[2]ULOQ: Upper Limit of Quantification, defined to be at least two times higher than the highest expected MTT formazan concentration in isopropanol extracts from negative controls

Reference

- US FDA (2001)
  U.S. Department of Health and Human Services, Food and Drug Administration.Guidance for Industry: Bioanalytical Method Validation. Available at: [http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf].