# Supporting information

# Desorption/ionization-MS methods for drug quantification in biological matrices and their validation following regulatory guidance.

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**Table of content**



**Abbreviations**

BMV, bioanalytical method validation ; BV, blind value ; CAL, calibration standard ; CoA, certificate of analysis ; CV, coefficient of variation ; DBS, dried blood spots ; DESI, desorption electrospray ionization ; DI, desorption/ionization ; EMA, European Medicines Agency ; FDA, US Food and Drug Administration ; ICH, International Committee for Harmonisation ; IS, internal standard (SIL-IS, stable-isotopically-labelled IS) ; ISR, incurred sample reanalysis ; LBA, ligand binding assay ; LC, liquid chromatography ; LOQ, limit of quantification (LLOQ, lower LOQ ; ULOQ, upper LOQ) ; MALDI, matrix-assisted laser desorption/ionization ; MF, biological matrix effect ; MS, mass spectrometry (MS/MS, tandem MS) ; QC, quality control (LQC, low QC ; MQC, middle QC ; HQC, high QC) ; SOP, standard operating procedure.

# I. Bioanalytical method validation

In this part are provided the compiled guidelines and practical aspects of bioanalytical method validation (BMV) of chromatography-based assay according to the US Food and Drug Administration (FDA), the European Medicines Agency (EMA), and the International Committee for Harmonization (ICH) requirements [1-3]. These are complemented with adaptations and proposals for the BMV of desorption/ionization (DI)- mass spectrometry (MS) methods.
All added paragraphs specific to guidelines for DI-based assay validation are suggestions from the authors and should be taken as possible lines of approach to implement as it is or to adapt depending on the specific requirements of the developed assay.

**Table of contents for BMV**

## 1. Introduction

Bioanalytical method validation is essential to ensure the acceptability of the assay performance and the reliability of analytical results.
Analytical methods, which are either new methods or adapted or modified methods from existing (described) methods, must be validated prior to their application to the analysis of medicinal products in biological material.
Bioanalytical method should be validated for each analyte in the case of multiplexed methods. Validation should be carried out in the same biological matrix as the study samples, including anti-coagulants and additives, except for rare matrices in which case a carefully selected surrogate biological matrix can be used.

The following standard operating procedure (SOP) describes the procedure for validating new, transferred, or modified liquid chromatography (LC)-MS or DI-MS methods. It represents the case of the most extensive validation. The quality of the analysis is described by various parameters, which are defined in this document and determined with the help of calibration standard (CAL) and quality control (QC) samples.

## 2. Summary of regulatory guidelines for bioanalytical method validation and suggested adaptations for desorption/ionization methods

Table S1 summarizes the different parameters (key points for sample preparation and acceptance criteria) for a full method validation given by the regulatory agencies for chromatography-based and ligand-binding assays, together with suggested adaptations for the validation of DI-based bioanalytical assays.

**Table S1.** Summary of the different steps for bioanalytical method validation in the frame of a clinical study as described in the regulatory guidelines for chromatography-based assays with suggested adaptations for desorption/ionization-based assays.

| | **Guidelines for chromatography-based bioanalytical assays Criteria** | **Suggested adaptations for desorption/ionization methods** |
|---|---|---|
| **Calibration (See 3.4)** | - At least six non-zero calibration levels from LLOQ to ULOQ, and CAL 0 and BV samples.<br>- 3 accepted calibration curves (excluding BV and CAL 0) with at least $r^2$=0.98.<br>- Accuracy on back calculated concentrations: ±15% bias or ±20% bias at LLOQ.<br>- At least 75% of non-zero calibration samples on at least six concentration levels should be accepted.<br>- At least 50% of replicates of each level should be accepted. | Same workflow, criteria, and actions. |
| **Accuracy and precision (See 3.5)** | - At least six replicates at the LLOQ level and at least three QC levels: LQC ≤ 3xLLOQ, MQC = 30-50% calibration range, and HQC ≥ 75% ULOQ.<br>- At least three accuracy and precision batches performed on at least 2 different days.<br>- Within-run and between-run accuracy: ±15% bias, or ±20% bias at LLOQ.<br>- Within-run and between-run precision: 15% CV, or 20% CV at LLOQ. | Same workflow, criteria, and actions. |
| **Specificity (See3.6)** | - At least six BV samples from at least six different individual sources (not pooled) and at least one BV sample from 1 individual (not pooled) for lipemic, haemolized and special population matrix selectivity.<br>- Analyte signal in BV samples: < 20% of the lowest LLOQ signal of the analyte.<br>- IS signal in BV samples: < 5% of the lowest IS signal in QC and calibrators. | Same workflow, criteria, and actions.<br>In some cases for tissue analysis, it might not be possible to obtain tissue from six individual sources. In such cases, specificity assessment can be performed on tissues from three individual sources. |
| **Carry over (See 3.9)** | - Two eluent samples analyzed after the highest calibration level.<br>- Analyte signal in eluent samples: < 20% of the lowest LLOQ signal of the analyte.<br>- IS signal in eluent samples: < 5% of the lowest IS signal in QC and calibrators. | - *MALDI-MS analyses of liquid samples:* The MALDI metal target should be washed after analyses using $H_2O$ and organic solvents, then eluent samples should be deposited on the same spots where the highest CALs or QCs were previously deposited to verify that the washing process enables to prevent carry-over between batches.<br>- *DESI-MS analyses*: Carry-over due to possible contamination of the DESI nozzle should be tested as for LC-MS-based assays.<br>- *MALDI-MS analyses of tissue sections deposited on glass slides:* No carry-over experiment is needed. |

| | Guidelines for chromatography-based bioanalytical assays<br>Criteria | Suggested adaptations for desorption/ionization methods |
|---|---|---|
| **Biological matrix effect and recovery (See 3.7 ;3.8)** | *Recovery*:<br>- Three replicates for each QC level.<br>- Recovery should be reproducible between replicates and QC levels.<br><br>*Biological matrix effect (MF, $MF_{IS}$; $MF_{norm}$)*:<br>- Three replicates on at least low and high QC levels.<br>- Precision on $MF_{norm}$ for each QC level: < ±15%.<br><br>*Both*:<br>- Blank biological matrix from at least six different individual sources (not pooled).<br>- Blank biological matrix is spiked with analyte and IS post-extraction (100%-QC for Recovery).<br>- Buffer is spiked with analyte and IS post-extraction (Eluent-QC for MF). | *Recovery*:<br>- The need for recovery assessment must be evaluated depending on the developed workflow.<br>- MALDI/DESI-MS of biological fluids using i) no sample preparation or biological matrix dilution = no recovery evaluation, ii) extraction and direct deposition = recovery assessment using adapted 100%-QCs (QC REC)[4] and a specific diluted QC series (QC DIL)[4], iii) extraction and drying = recovery evaluation similar to LC-MS/MS.<br>- DESI-MS analyses of DBS: if the IS is: i) impregnated in DBS paper before blood collection = no recovery assessment needed, or ii) deposited on sample before analysis = recovery evaluation similar to LC-MS/MS.<br>- MALDI/DESI-MS of tissues: i) recovery evaluation to compare normal QCs (standards deposited externally) with tissues dosed *in vitro* (immersion in QC solutions, mimetic QCs), ii) recovery assessment of the extraction occurring when depositing/spraying IS and MALDI matrix solutions.<br><br>*Biological matrix effect*:<br>- In general, the biological matrix effect can be assessed using DI methods similarly to LC-MS/MS methods.<br>- For DI-MS assays for liquid biological matrices processed with an extraction step followed by direct analyses, additional sets of QCs must be prepared to overcome dilution effects and signal variability due to surface effects (QC DIL[4], QC MAT[4], and QC EL[4]).<br>- MS-based assays for DBS analyses: additional parameters must be controlled, such as hematocrit effect and paper carrier effect.<br><br>*Both*:<br>- In some cases for tissue analysis, it might not be possible to obtain tissue from six individual sources. In such cases, recovery and biological matrix effect assessment can be performed on tissues from at least three individual sources. |
| **Dilution integrity (See 3.10)** | - QC samples above ULOQ and dilution according to dilution schemes used for study samples.<br>- At least five replicates on each required dilution levels (six if batch for partial validation).<br>- Accuracy at each dilution level: ±15% bias.<br>Precision: 15% CV. | Same workflow, criteria, and actions. |

| | Guidelines for chromatography-based bioanalytical assays<br>Criteria | Suggested adaptations for desorption/ionization methods |
|---|---|---|
| **Stability tests (See 3.11)** | - At least three replicates at low and high QC levels for each stability time/temperature point.<br>- Prepared QCs analyzed once at t=0 and at the different time points.<br>- Fresh QC samples and calibration standards for each stability batch.<br>At each time/temperature point:<br>- Accuracy within ±15% bias.<br>- Precision below 15% CV. | Same workflow, criteria, and actions. |
| **Analytical runs (See 4)** | - CAL 0 + ≥ six accepted non-zero calibration levels from LLOQ to ULOQ (≥50% of accepted replicates per level)<br>- Overall ≥ 75% of calibration within ±15% bias accuracy limits (except CAL at LLOQ: ±20% bias).<br>- BV and eluent samples for selectivity and carry-over check on each run.<br>- At least three accepted QC levels (LQC, MQC, and HQC). QC number covering 5% of total number of study samples, with a minimum of six QC samples (three levels in duplicates). QC placed throughout the batch in alternation with study samples.<br>- At least 67% of QC samples. Within-run and between-run accuracy within ±15% bias and within-run and between-run precision below 15% CV.<br>- All samples from one individual should be processed within the same analytical run and if possible on the same plate. | Same workflow, criteria, and actions. |
| **ISR (See 4.5)** | - 10% of the first 1000 samples + 5% of the remaining samples.<br>- ISR samples around $c_{max}$ and in the elimination phase, representative of the complete sample set.<br>- Samples with original concentration ≥ 3xLLOQ and ≤ 75% ULOQ.<br>- At least 67% of repeated values should be within ±20% of the mean.<br>- If repeated concentrations are below LLOQ or above ULOQ, excluded from ISR calculations. | Same workflow, criteria, and actions. |
| **Cross-validation (data bridging) (4.6)** | If cross-validation is required, criteria for ISR should be applied. | - As quantification DI assays are still not very common, it might be necessary for each new fully validated assay to perform a cross-validation with another validated gold-standard quantification method (e.g. LC-MS/MS or LBA assay).<br>- In any case, if the full validation is not possible for DI assays, the method must be validated at least using data bridging with another established analytical approach.<br>- Cross-validation of results using samples dosed *in vivo* or *in vitro* might also be useful during method development to make sure that the developed DI assay yield similar results than LC-MS/MS (for instance) before starting any validation, including for the preparation of CAL and QC samples. |

## 3. Method validation procedure and requirements

The nomenclature and procedures of this standard operating procedure (SOP) are in accordance with the guidelines proposed by the FDA (Guidance for Industry - Bioanalytical Method Validation, May 2018), the EMA (Guideline on Bioanalytical Method Validation, 2011), and the ICH (Bioanalytical Method validation M10, Draft version. March 2019) [1-3] Each section corresponds to a different validation parameter and can be read separately from the others.

### 3.1. Method description

The extended description of the method stands as the basis of the validation. This should be written so that an experienced analyst is able to understand the method in all its steps on the basis of it alone.

### 3.2. Reference standards, blank matrices

**Reference standards**

- Analytes of interest and internal standards (IS) used as reference substances should be provided as commercially available standards, pharmaceutical standards, or synthesized standards. A certificate of analysis (CoA) or equivalent should be provided for each reference substance with full information regarding purity, storage conditions, retest/expiration date, and batch number.
- The reference standard should be well characterized (identity, purity, etc.) and identical to the analyte of interest, or at least of an established form (salt, hydrate, etc.) of known quality.
- Stock solution should be used only within the proven stability period. If reference standards have expired, identity and purity need to be reestablished, as well as delay before next reference test.
- The IS does not necessitate a CoA as long as the suitability for use is demonstrated (lack of interference with the analyte of interest or lack of impurities). The use of a stable isotopically labeled (SIL) IS is recommended but not mandatory. In the case of a SIL-IS, high purity and no isotope exchange should be ensured (i.e. use of C13- and N15-labeled standards instead of D-labeled is therefore recommended). If another type of IS is chosen, this should be justified.

**Blank matrices**

- For method validation, blank matrices should be recorded using batch/lot number, date of collection, storage conditions, and date of use.
- The selected blank biological matrix should be the same as the biological matrix of the study samples. If not, at least one precision and accuracy batch should be performed in the same biological matrix as the study samples.
- If needed in the case of rare biological matrices, a surrogate biological matrix can be used for calibration and QC samples. In such cases, the choice of the surrogate biological matrix should be justified and the biological matrix effect of the method should be tested in both the biological matrix (only three different individual sources) and the surrogate biological matrix. Selectivity in the real biological matrix should also be proven in blank samples from only three different individual sources.

### 3.3. Labeling and documentation

**Labeling**

The bottles and vials containing the reference substances should be marked with permanent labels stating the maximum of identification information, including dates of manufacture and expiration.

**Documentation**

- The information required to identify and keep track of the analytical reference substances and blank matrices, such as provider, nature, batch number, order number, storage condition, date of use, amount used, etc., should be documented on-site and in validation and analytical reports. If not included, certificates of analysis must be requested and filed from the manufacturer.

### 3.4. Calibration curve and calibration range

**General considerations**

*Definition*

A calibration solution is an aqueous and / or organic solution that contains the targeted analyte dissolved in a certain concentration. Calibration standard samples (CALs) are prepared by adding a certain volume of the calibration solutions to the biological matrix (e.g. plasma). The calibration curves are determined from the CALs. A calibration curve describes the correlation between the concentration of the analyte and the "response" obtained from the measuring device. It serves to quantify clinical samples of unknown concentration.

*Aim*

Check for the linearity and the reliability of the normalized response (ratio of analyte peak area over IS peak area) within the concentration range of interest. If more than one analyte are targeted into the clinical samples, one calibration curve must be done for each of them.

*Key aspects*

- The concentration range of the calibration curve depends on the concentrations expected for the administered dose. The calibration point with the highest concentration ("upper limit of quantification" – ULOQ) should be approximatively 30% above the expected maximum concentration or should be the highest concentration in the proven linearity range of the method. The calibration point with the lowest concentration ("lower limit of quantification" – LLOQ) is determined during method development and before method validation. The LLOQ signal should be at least five times higher than the analyte signal obtained in the zero-level calibration standard (CAL 0 – IS only in biological matrix).
- The CALs should include: blank biological matrix with no analyte and no IS (BV – blind value), blank biological matrix with only IS as zero-level calibrator (CAL 0), and blank biological matrix spiked with increasing concentrations of analyte and constant concentration of IS from LLOQ to ULOQ. The calibration curve should comprise at least six non-zero concentration levels. Blank samples and each CAL level can be prepared in replicates as needed.
- The preparation of the calibration solutions depends on the targeted analyte and the state of the available reference compound. The full preparation workflow for stock and calibration solutions, as well as for CALs, should be predefined in a SOP before method validation and should stay the same over the complete validation-analytical process.
- CALs should be prepared in the same biological matrix as the clinical samples.

**Sample preparation for LC-MS-based assays**

*Preparation of the calibration solutions*

- In general, in the case of small molecule drugs or peptide-based drugs, the stock solution is obtained from a lyophilized powder of the reference compound. This should be precisely weighted and dissolved using an accurate volume of solvent (preferably $H_2O$ or aqueous dilutions of $H_2O$/miscible organic solvents if the poor solubility requires it – eg. 1:1 v/v ACN/$H_2O$ or MeOH/$H_2O$).

In the case of antibody-based drugs (eg. monoclonal antibodies – mAb), for which it can be difficult to obtain lyophilized powder of the reference compound, a highly concentrated and precisely characterized solution can also be used as stock solution.

In all cases, in order to obtain calibration solutions of different concentrations, serial dilutions should be made from the stock solution if possible. Dilutions can be carried out with accurate volume measurement tools, such as calibrated reciprocating pipettes, full pipettes, and corresponding volumetric flasks, and should mainly contain water or water-miscible organic solvents.

- The stock solution and calibration solutions should be prepared fresh before each batch or stored under defined conditions, for which the stability over time was fully tested. In case of preparation of a new stock solution or a new set of calibration solutions, the quality of the new solutions should be tested following a predefined SOP and the solutions should be used only after agreement.

*Preparation of the calibration standard samples*

- The CALs are usually prepared by adding a defined volume of the calibration solutions to the biological matrix (e.g. plasma). In exceptional cases, e.g. when no biological matrix is available, CALs can be prepared using suitable surrogate biological matrix (buffer solutions for instance). In such case, the choice of the surrogate biological matrix should be justified and the correlation with the original biological matrix proven (see 3.2).

  Particular attention should be paid to the following points:

  1. CALs should be prepared fresh before each batch as long as no stability experiments in the biological matrix was performed to prove stability of analyte and IS under the storage conditions. If not possible, at least one batch should respect this point. In any case, fresh CALs should always be preferred and their storage should always be justified.

  2. The volume of the calibration solution that is added to the empty biological matrix should be as small as possible (e.g. 25 µL calibration solution to 100 µL empty biological matrix).

  3. The added volume of the calibration solution (e.g. 25 µL) should be the same for each calibration sample, regardless of the concentration of the calibration solution.

- The number and amount of calibration solutions available for the production of the calibration samples should be sufficient to fully cover the calibration range and enable the production of replicate calibration samples as needed. In addition, calibration solutions and CALs, as well as QC solutions and samples should be produced together. If there are not enough CALs available for the analyses, a full set of new CALs may be produced.

*Labeling*

- The vials containing the calibration solutions and samples should be marked with permanent labels stating all information important for identification such as :
  1. Substance name or possibly abbreviation.
  2. Sample reference (BV, CAL 0, CAL A…)
  3. Substance concentration
  4. Solvent and original volume used.
  5. Date of manufacture
- Reference codes of CALs should be chosen as clearly as possible. For instance, with CAL A, CAL B, etc., where the letters (A, B, …) describe the calibration samples in increasing concentration starting with CAL A as the LLOQ, or with CAL followed by the concentration of the designed level (e.g. CAL 5, CAL 20, …, CAL 1000, where 5 would designate a LLOQ at 5 ng/mL and 1000 a ULOQ at 1000 ng/mL for instance). Blank biological matrix without analyte and IS is labeled as BV and blank biological matrix with only IS is labeled as CAL 0.

**Sample preparation for DI-based assays**

The overall sample preparation procedures are generally similar for DI-based assays as for LC-MS-based assays, especially when talking about liquid biological matrices (e.g. blood, plasma, urine).

For dried blood spot (DBS) and tissue sections however, the procedure for the creation of CALs requires careful consideration and optimization.

Studied samples have been impregnated with the drug of interest *in vivo* and it is therefore important to develop a spiking/extraction process for CALs that will realistically reproduce what is happening with dosed samples during the sample preparation process. Before validating a DI-based bioanalytical assay, it is therefore crucial to fine-tune the creation of the CALs. For instance, in order to choose the final approach for BMV, different strategies might be tested (automatic or manual deposition, mixing of reference standard and IS with MALDI matrix before deposition, deposition of reference standard and IS followed by deposition of the MALDI matrix, etc. [5]) by analyzing tissues dosed *in vitro* and comparing the obtained results to LC-MS-MS quantification results of the same tissues.

**Calibration calculations and acceptance criteria**

*Calibration model*

- BV and CAL 0 samples should not be taken into account for the calculation of the curve parameters.
- The simplest possible model should be taken into account for the calculations of the calibration curve parameters (e.g. linear regression, 1/x weighting). The use of more complex regression model has to be justified. The regression model and weighting scheme should stay the same throughout the validation-analytical process.

*Acceptance criteria*

- At least three calibration curves must be accepted on at least two different days during method validation.
- Back calculated concentration accuracy calculated as a percentage of deviation (See 3.5, Equation (1)) should be within ±15% bias when compared to expected concentrations, except for the LLOQ which should be within ±20% bias.
- A minimum of 75% of the calibration points on at least six concentration levels must meet these criteria. At least 50% of each technical replicate must also fulfill these criteria.

*Action if failing criteria*

- Data points that do not meet the criteria (and only these) should be excluded from the calculation and the regression model.
- If all replicates of one calibration level are rejected, linear regression and back calculations must be reevaluated without this level and model should not be modified.
- If LLOQ or ULOQ levels are excluded then the batch must be rejected.
- If one batch is rejected (more than 75% of calibration points are rejected, LLOQ or ULOQ levels are rejected, or less than six non-zero concentration levels are accepted), possible source of failure must be determined and method revised as needed. If next batch is also rejected, method must be revised and validation restarted.

## 3.5. Accuracy and precision

**General considerations**

*Definition*

Similar to the CALs, a QC solution is an aqueous and / or organic solution in which the targeted analytes are dissolved to reach a certain concentration. QC samples are empty matrices (e.g. plasma) spiked with defined concentrations of the targeted analyte. QC samples are quantified in each analytical batch to

evaluate the quality of the analysis by calculating within- and between-run accuracy and precision parameters.

*Aim*

The accuracy of an analytical method describes the closeness of the determined value obtained by the method to the expected concentration of the analyte (expressed in percentage of deviation – % bias).

$$Accuracy\ [\%\ bias] = \frac{Calculated\ concentration - Expected\ concentration}{Expected\ concentration} \times 100 \qquad (1)$$

The precision of the analytical method describes the closeness of repeated individual measures of the analyte. Precision is expressed as the coefficient of variation (% CV).

$$\%\ CV = \frac{\sigma}{\mu} * 100 \qquad (2)$$

Where $\sigma = \sqrt{\frac{\Sigma(x-\mu)^2}{n-1}}$ corresponds to the standard deviation

and $\mu = \frac{\Sigma x}{n}$ corresponds to the arithmetic mean.

*Key aspects*

- Within the scope of a method validation, QC samples should be produced in a minimum of four concentrations: LLOQ, low QC (LQC) ≤ 3*LLOQ, medium QC (MQC) = 30-50% of calibration curve range and high QC (HQC) ≥ 75% of ULOQ.
- As for CALs, a constant IS amount should be added in all QC samples for internal normalization purpose.
- A minimum of five technical replicates should be prepared for each QC level.
- As for calibration solutions (see 3.4), the preparation of the QC solutions depends on the targeted analyte and the state of the available reference compound. The full preparation workflow for stock and QC solutions, as well as for QC standards, should be predefined in a SOP before method validation and should stay the same over the complete validation-analytical process.
- QC standards should be prepared in the same biological matrix as the calibration and clinical samples.

**Sample preparation for LC-MS-based assays**

*Preparation of the quality control solutions*

- QC solutions should be prepared from a different stock solution than the calibration solutions. If accuracy and precision were proven on one validation batch then the same stock solution can be used for the following.
- As for calibration solutions, in general, in the case of small molecule drugs or peptide-based drugs, the QC stock solution is obtained from a lyophilized powder of the reference compound. This should be precisely weighted and dissolved using an accurate volume of solvent (preferably $H_2O$ or aqueous dilutions of $H_2O$/miscible organic solvents if the poor solubility requires it – eg. 1:1 v/v ACN/$H_2O$ or MeOH/$H_2O$).

  In the case of antibody-based drugs (eg. monoclonal antibodies – mAb), for which it can be difficult to obtain lyophilized powder of the reference compound, a highly concentrated and precisely characterized solution can also be used as QC stock solution.

  In all cases, in order to obtain QC solutions of different concentrations, serial dilutions should be made from a starting solution, mentioned above as "QC stock solution", if possible. Dilutions can be carried out with accurate volume measurement tools, such as calibrated reciprocating pipettes, full pipettes, and corresponding volumetric flasks, and should mainly contain water or water-miscible organic solvents.

- The QC stock solution and QC solutions should be prepared fresh before each batch or stored under defined conditions, for which the stability over time was fully tested. In case of the preparation of a new stock solution or a new set of QC solutions, the quality of the new solutions should be tested following a predefined SOP and the solutions should be used only after agreement.

*Preparation of the quality control samples*

- As for calibration samples, the QC samples are usually prepared by adding a defined volume of the QC solutions to the sample matrix (e.g. plasma). In exceptional cases, e.g. when no biological matrix is available, QC samples can be prepared using suitable surrogate biological matrix (see 3.2). Particular attention should be paid to the following points:
  1. QC standards should be prepared fresh before each batch as long as no stability experiments in the biological matrix was performed to prove stability of analyte and IS under the storage conditions. If not possible, at least one batch should respect this point. In any case, fresh QC samples should always be preferred and their storage should always be justified.
  2. The volume of the QC solution that is added to the empty biological matrix should be as small as possible (e.g. 25 µL QC solution to 100 µL empty biological matrix).
  3. The added volume of the QC solution (e.g. 25 µL) should be the same for each QC sample, regardless of the concentration of the QC solution.
- The number and amount of QC solutions available for the production of the QC samples should be sufficient to fully cover the four concentration levels and enable the production of replicate QC samples as needed. In addition, calibration solutions and CALs, as well as QC solutions and samples (see 3.4) should be produced together. If there are not enough QC samples available for the analyses, a full set of new QC solutions may be produced.

*Labelling*

- The vials containing the QC solutions and samples should be marked with permanent labels stating all information important for identification such as :
  1. Substance name or possibly abbreviation,
  2. Sample reference (e.g. LLOQ, LQC, MQC, and HQC),
  3. Substance concentration,
  4. Solvent and original volume used,
  5. Date of manufacture.

**Sample preparation for DI-based assays**

The overall sample preparation procedures are generally similar for DI-based assays as for LC-MS-based assays, especially for liquid biological matrices (e.g. blood, plasma, urine).

For DBS and tissue sections however, the procedure for the creation of QCs requires careful consideration and optimization.

Studied samples have been impregnated with the drug of interest *in vivo* and it is therefore important to develop a spiking/extraction process for QCs that will realistically reproduce what is happening with dosed samples during the sample preparation process. Before validating a DI-based bioanalytical assay, it is therefore crucial to fine-tune the creation of the QCs. For instance, in order to choose the final approach for BMV, different strategies might be tested (automatic or manual deposition, mixing of reference standard and IS with MALDI matrix before deposition, deposition of reference standard and IS followed by deposition of the MALDI matrix, etc. [5]) by analyzing tissues dosed *in vitro* and comparing the obtained results to LC-MS-MS quantification results of the same tissues.

**Parameter calculations and acceptance criteria**

*Parameter calculations*

- Within- and between-run accuracies and precisions should be proven in a minimum of three batches carried out on at least two different days.
- Accuracy and precision calculation for validation should include all obtained data, including those outside of acceptance criteria, except when errors are obvious and documented.
- To enable evaluation of any trends over time within one run, it is recommended to demonstrate accuracy and precision of QC samples over at least one batch in a size equivalent to a prospective run of clinical samples.

*Acceptance criteria*
- Within-run and between-run accuracies should be within ±15% bias of the nominal value for all QC levels, except for LLOQ which should be within ±20% bias.
- Within-run and between-run precisions should be within 15% CV for all QC levels, except for LLOQ which should be within 20% CV.

*Action if failing criteria*
- If within-run accuracy and/or precision is out of the criteria on one QC level, the batch should be rejected.
- If one batch is rejected, possible source of failure must be determined and method revised as needed. If next batch is also rejected, method must be revised and validation restarted.
- Method cannot be validated until between-run accuracy and precision are falling into the validation criteria.

### 3.6. Selectivity/specificity

**General considerations**

*Aim*

Check if the analyte of interest and IS are easily discriminable from endogenous compounds or other needed compound before or during sample collection (stabilizers, enzyme, different coagulants, co-medication, etc.).

*Blank biological matrix choice for selectivity control*
- Test of selectivity in six blank matrices (BV) from six different individual sources (no pooled biological matrix) similar to the clinical sample matrix. In the case of rare biological matrix, less individual sources might be used, but there must be a minimum of three biological matrices from three individual sources.
- When needed, lipemic, haemolized matrices, or matrices from special population, from at least one individual (no pooled biological matrix) should also be tested.
- When needed, selectivity should be assessed by testing blank matrices in the presence of co-medication, critical reagents or other exogenous molecules that could interfere with the signal of the analyte of interest.

**Acceptance criteria and corrective measures**

*Acceptance criteria*

Absence of interfering components is validated when the signal in BV samples is less than 20% of the lowest LLOQ signal for the analyte and less than 5% of the average signal for the IS in QC samples and CALs.

*Action if failing criteria*
Sample preparation, reference standards, LLOQ and/or LC-MS methods should be changed or optimized to fall into the acceptance criteria for selectivity.

### 3.7. Recovery

**General considerations**
*Aim*
Check if the sample preparation enables a reproducible, accurate recovery of the analyte and IS.

*Nomenclature*
QC: QC samples prepared following the normal sample processing workflow.
100%-QC: QC samples with analyte and IS spiked post-extraction in the blank biological matrix

*Key parameters for recovery testing*
- Recovery should be determined on each QC level in triplicate.
- Blank biological matrix from at least six individual donors (no pooled biological matrix) should be used. In the case of rare biological matrix, less individual sources might be used, but there must be a minimum of three biological matrices from three individual sources.
- QC samples of each concentration levels are prepared in triplicates in the blank biological matrix (usually three QC samples from the precision and accuracy QC samples are selected for recovery calculation) and additional QC samples are prepared in triplicates by spiking the analyte post-extraction step in the blank biological matrix.

**Specific sample preparation for DI-based assays**
The recovery measurements when using DI-based assays depends on the biological matrix and the sample preparation process applied.

*DI-MS analyses of liquid biological matrices*
In the case of liquid biological matrices (e.g. blood, plasma), the assessment of the recovery efficiency and reproducibility depends on two strategies:
- No extraction is performed: The biological samples are deposited with the MALDI matrix onto the MALDI target plate, sometimes with a dilution step. In DESI, the biological sample can be deposited on glass slide and directly analyzed. No extraction occurs; therefore no recovery assessment is required.
- Sample preparation relies on an extraction step: To simplify the biological background and improve the sensibility of the DI-based assay, it is often useful to perform an extraction step (e.g. liquid liquid extraction) before the analysis, and then recovery assessment will be needed for BMV.
  i) If the samples preparation follows a similar workflow as for LC-MS/MS assay (extraction, drying, and resuspension), then the recovery assessment procedure is similar as for LC-MS/MS assays.
  ii) If a more rapid workflow is used, with extraction step and direct deposition of the extract on the DI support, then some adaptations are required (Supporting information, section II.1., **Figure S1**). Similarly to LC-MS/MS assay, the QC for recovery (QC-REC) are prepared by spiking the sample with the reference standard and the IS after the extraction step (**Figure S1c.**). However, because the extract is not dried, there is a dilution effect when adding the standard solutions. To correct this during recovery experiments, it is necessary to create diluted QC series (QC DIL, **Figure S1b**) that will be used for comparison with QC REC series and recovery calculations.

When using DI methods, there is a signal variability induced by surface effects (due to sample surface, as well as crystal morphology in MALDI experiment) that can be significant even between technical or analytical replicates. Because of this, for recovery experiment, normalization should be applied using the IS. Contrarily to LC-MS/MS assays, the IS should not be added post-extraction with the reference standard when creating the 100%-QC or QC REC series, but it should be added pre-extraction (when IS solution does not induce the extraction), to enable similar normalization as for the normal QC or QC DIL series and correct the signal variability (**Figure S1.c**).

*DESI-MS analyses of dried blood spots*

As desorption electrospray ionization (DESI) method enables the direct analysis of biological samples without sample preparation, there is in theory no extraction process before analysis and therefore no recovery experiment would be needed. This is particularly true when the IS is deposited on the DBS support before blood collection.

However, in many cases, the IS solution has to be deposited/sprayed on top of the DBS sample, which acts as an extraction step (Supporting information, section II.2., **Figure S2a and S2d**). In such case, it would thus be necessary to assess the recovery efficiency of this extraction step and its reproducibility during method validation. For this, similarly to LC-MS-based assays, the QC samples used for accuracy and precision measurements should be compared to 100%-QCs prepared by depositing on the blank biological matrix (i.e. blood) the reference standard and IS solutions mixed together (**Figure S2b**).

*MALDI/DESI-MS analyses of tissues*

For tissues, two different recovery coefficients should be taken into account and evaluated separately during BMV.

- For tissue analyses, the IS and MALDI matrix are deposited/sprayed onto the dosed tissue. This creates an extraction effect of the endogenous compounds and the drug towards the tissue surface by the IS and MALDI matrix solutions. The efficiency and reproducibility of this extraction effect must thus be evaluated and proven during BMV, as for a classical extraction process. For the quantification of tissues using DI methods, different strategies can be applied for the preparation of CALs and normal QCs (**Figure S3b**): i) blank tissue sections spiked by manual deposition or by automatic spotting/spraying of a reference standard solution at different concentrations to create CALs and QCs, ii) tissue homogenates spiked by manual deposition or by automatic spotting/spraying of a reference standard solution at different concentrations to create CALs and QCs, iii) tissue homogenates spiked with small amount of CAL and QC solutions, cryosectioned, and mounted on glass slides, and iv) tissue pieces immersed in CAL and QC solutions for several hours, cryosectioned, and mounted on glass slides. IS solution is subsequently deposited, followed by MALDI matrix (for MALDI-MS assays). For recovery experiment, the reference standard solution should be mixed and deposited either with the IS solution (DESI-based assay) or with the MALDI-matrix solution (MALDI-based assay) to create the 100%-QC series (**Figure S3c**). The created 100%-QC series is then compared to the normal QC series for recovery calculations.
- Using external deposition/spraying of the reference standard solution on blank tissue sections or on sections of tissue homogenates to create the CALs and QCs for drug quantification are reliable strategies to accurately control the targeted compound concentrations in biological matrix and enable obtaining accurate and reproducible quantification results. However, these strategies create some discrepancies in the overall workflow and in the sample morphology when compared to the sample preparation process for tissues dosed *in vivo* (**Figure S3e**), because the drug is already incorporated in the biological samples and not added on the section. Because of this, a second recovery coefficient ("*mimetic* recovery") must be here evaluated when using external deposition of reference standard to make sure that the applied strategy for CAL and QC preparation allows retrieving a reproducible and optimal extraction when compared to real samples (**Figure S3a** and **Figure S3b**). This *mimetic* recovery evaluation might then be used as a correction factor for a more

accurate quantification. Moreover, as the classical recovery experiment, this evaluation must be performed at different concentration levels and for biological matrices from six different individual sources (or at least three different individual sources for rare biological matrices).
Regarding the experimental realization, tissues should first be dosed *in vitro* (e.g. by immersing a blank tissue into a reference standard solution, or by spiking a tissue homogenate with a reference standard solution). The tissue dosed *in vitro* must be cryosectioned and prepared using the developed workflow for the DI assay (i.e. IS deposition followed by MALDI matrix deposition if required) to create the "mimetic" QC series (QC MIM, **Figure S3a**). In parallel, normal QC series (external deposition of the reference standard or use of tissue homogenates) at the observed concentrations of the QC MIM (determined during method development using LC-MS/MS, when *in-vitro* dosed tissues are created by immersion in standard solution) should be prepared and measured. Mimetic recovery coefficient for the extraction of the drug is then calculated by comparison between QC MIM and normal QC series. It is important to note that this mimetic recovery evaluation is only useful when normal QCs and CAL are created by external deposition of standards and IS (e.g. by spraying or spotting methods).

As for recovery evaluation during DI-MS analysis of liquid biological matrices, the step of IS addition to the sample must not be changed between samples compared for recovery calculations in order to normalize signals and correct variabilities coming from surface effects (i.e. IS always added pre-extraction or during extraction).

**Acceptance criteria and corrective measures**
*Acceptance criteria*
Recovery on all three QC levels does not need to be 100% but should be reproducible.

*Action if failing criteria*
Reasons for irreproducibility should be investigated and corrected.

3.8. Biological matrix effect (MF)

**General considerations**
*Aim*
Check whether or not the biological matrix influences the response of the analyte or the IS.

*Nomenclature*
100%-QC: QC samples with analyte and IS spiked post-extraction in the blank biological matrix
Eluent-QC: QC samples with analyte and IS spiked post-extraction in the buffer
MF: Biological matrix factor of the analyte
$MF_{IS}$: Biological matrix factor of the internal standard
$MF_{norm}$: Normalized biological matrix factor

*Key parameters for biological matrix effect*
- Biological matrix effect of both analyte of interest and IS should be determine on at least low and high QC levels in at least triplicates.
- Usually 100%-QC prepared for recovery experiments are also used for biological matrix effect calculations.

- As for recovery experiments, blank biological matrix from at least six individual donors (no pooled biological matrix) should be used. If six lots cannot be used, lower number of different lots can be used but this must be justified (rare biological matrix).
- If critical reagents (co-medication, compounds from formulation, enzymes, …) are present in the biological matrix, MF should be calculated in presence of these compounds. In these cases, blank biological matrix should be obtained from individuals who received these excipients, except if this has been proven that no metabolization or transformation occurs in vivo.
- Eluent-QC samples are prepared in triplicates by spiking the analyte and IS post-extraction step in the buffer.
- MF of the analyte is then calculated as described in (3) :

$$\mathrm{MF} = \frac{\text{Peak area}_{(\text{analyte in } 100\% - \text{QC})}}{\text{Peak area}_{(\text{analyte in Eluent} - \text{QC})}} \qquad (3)$$

- $MF_{IS}$ is calculated in the same way using IS peak area.
- Normalized MF is then calculated as in (4) :

$$MF_{norm} = \frac{MF}{MF_{IS}} \qquad (4)$$

**Specific sample preparation for DI-based assays**

The overall sample preparation procedures for biological matrix effect evaluation are generally similar for DI-based assays as for LC-MS-based assays and an Eluent-QC series must be created to be compared with the 100%-QC series produced for recovery evaluation. (**Figure S2c**, **Figure S3d**).

Regarding DI-MS assays based on the drug extraction followed by direct analysis of the extract, some adaptations are required before measuring the biological matrix effect. For this, two QC series should be prepared: the Eluent-QC series (**Figure S1e**), similarly to LC-MS/MS assays, and a QC MAT series consisting of blank biological matrix extracted following the developed workflow and subsequently spiked with the reference standard and IS solutions (**Figure S1d**). For the QC MAT, the initial volume of blank biological matrix must be increased in order to overcome the dilution effect due to the spiking of the standard solutions after extraction and maintain the same ratio drug/endogenous compounds as in the CAL, normal QC (**Figure S1a**), and clinical samples (**Figure S1f**), e.g. double volume of biological matrix for QC MAT if addition of standard solutions induces a 2-fold dilution.

**Specific matrix effect evaluation for dried blood spots analyses**

- For BMV of drug quantification in DBS, the guidelines recommend further matrix effect measurements, especially concerning the evaluation of the hematocrit effect (i.e. variability between biological samples due to changes in blood viscosity affecting the spreading of the blood on DBS paper, the spot homogeneity and the extraction recovery [6]), together with the assessment of sample homogeneity. This should be conducted similarly as biological matrix effect assessment by comparing Eluent-QCs with 100%-QC HTC (100%-QC hematocrit) series for different drug concentrations and in samples with different hematocrit values to evaluate the reproducibility of the hematocrit effect. More generally, this control should be applied to all assays using whole blood as biological matrix.
- For DESI-MS analyses of DBS, as well as for LC-MS/MS, an additional effect should also be tested: the paper carrier effect. Depending on the type of paper used as sample carrier for the blood collection, extraction of the drug might be favored or hampered, or sample dispersion might be increased, and thus variabilities can arise between similar samples collected on different paper carriers. This effect should thus be controlled in order to specify if the developed MS-based assay is applicable to DBS samples regardless of the type of carrier paper or if the studied DBS must all be collected on the same type of paper carrier. Such evaluation should then be performed similarly to

the biological matrix effect experiment, but with 100%-QCs all prepared in blood from the same individual source (not pooled) and on papier carriers of different types (ideally, all principal types of paper carriers used for sample collection, or at least all those described for the study of interest).

**Acceptance criteria and corrective measures**

*Acceptance criteria*

Precision on $MF_{norm}$ calculations from triplicates should not exceed 15% CV.

*Action if failing criteria*

Reasons for irreproducibility should be investigated and corrected.

### 3.9. Carry-over

**General considerations**

*Aim*

Check for no carry-over after highly concentrated samples, calibration standard or QC.

*Method for LC-MS/MS assays*

Analyze two eluent samples after the ULOQ calibration standard duplicates.

**Specific sample preparation for DI-based assays**

*MALDI-MS analyses of liquid samples*

The MALDI metal target should be washed after analyses using $H_2O$ and organic solvents, then eluent samples should be deposited on the same spots where the highest CALs or QCs were previously deposited to verify that the washing process enables to prevent carry-over between batches.

*DESI-MS analyses*

Similarly to possible carry-over from the LC column in LC-MS/MS assay, carry-over due to possible contamination of the DESI nozzle (entry stainless steel capillary directing the secondary droplets and formed ions into the mass spectrometer) should be tested similarly to LC-MS-based assays.

*MALDI-MS analyses of tissue sections*

As glass slides are single-use sample carriers, no carry-over should occur during MALDI-MS assays for drug quantification in tissue sections.

**Acceptance criteria and corrective measures**

*Acceptance criteria*

- Absence of carry-over is validated when the signals of the analyte and the IS in the eluent samples are less than 20% of the lowest signal of the CAL corresponding to the LLOQ or less than 5% for the lowest IS signal in the CALs.

*Action if failing criteria*

- If carry-over is detected, LC method should be revised as needed.
- If carry-over cannot be avoided, no randomization is possible and at least one eluent sample should be processed after each QC sample, study sample, or CAL expected to have a high analyte concentration.

### 3.10. Dilution integrity

**General considerations**

*Aim*

If study samples need to be diluted to fall into the linearity range of the method, dilution effect on accuracy and precision of the method should be investigated.

*Key parameters for dilution integrity testing*

- Prepare QC samples above the ULOQ and dilute them with defined aqueous solvent to fall in the calibration range.
- All dilution levels that are expected to be used for study samples need to be checked during method validation.
- Dilution integrity should be performed on at least five technical replicates for each dilution level.
- Dilution integrity can be covered by partial validation. In this case, six technical replicates per dilution level should be used.

**Acceptance criteria and corrective measures**

*Acceptance criteria*

- Accuracy at each dilution level should be within ± 15% of nominal concentration.
- Precision at each dilution level should be within 15% CV.

*Action if failing criteria*

- If dilution integrity cannot be proven, method should be revised and revalidated with an extended calibration range.

### 3.11. Stability tests

**General considerations**

*Definition*

The stability of a substance in biological materials depends on the sample matrix (e.g. plasma, cells, tissues), the anticoagulant present in the blood collection container (e.g. lithium heparin) and on the conditions at the storage location (e.g. temperature, light protection or air humidity). A substance is considered stable if the concentration changes by a maximum of ± 15% compared to the concentration at the start of the stability studies.

*Aim*

Stability experiments are used to test the stability of the analyte of interest and its internal standard during sample collection, handling and storage, in buffer and in biological matrix.

*Storage times*

The period for the stability examination should at least cover the conditions and the period of time corresponding to the clinical sample storage. If possible, however, the storage stability should be checked for at least 6 months. A review of stability should be examined at least at three different times. The starting point of the stability tests is to be documented in the analysis order belonging to the substance.

**Stability tests**

In principle, the conditions to be selected for a stability analysis are those to which the substance is exposed from sample collection to analysis. According to the general guidelines, the following stability tests are recommended:

1. Stock-solution stability: Stability of stock solutions of analyte and IS (concentrations used for storage) should be tested under the used storage conditions (e.g. -70°C, -20°C, or +4°C) at the appropriate concentrations for the analysis.
2. Bench-top stability: Short term stability of analyte and IS in biological matrix and buffer at room temperature within the time period covering the sample preparation if it is done at room temperature.
3. Autosampler stability: Short term stability in biological matrix at 10°C (in autosampler) for processed samples. Can be tested over 24h, 48h 72h or 1 week for instance.
4. Processed sample stability: If processed samples need to be stored before analysis or further processing, stability should be tested under the required storage conditions.
5. Freeze-and-thaw stability: Stability of analyte in the biological matrix without processing from storage conditions to room temperature. Check over at least three cycles. The freezing steps should last at least 12 hours before thawing.
6. Long term stability: Stability of analyte in biological matrix and, when meaningful, on sample carrier under storage conditions used prior to analyses (e.g. 20°C, 4°C, -20°C, or -70°C). This should be tested over a time period covering the time from the first sample collection to the last sample analysis.

**Key parameters for stability testing**

- Stability QC samples should be prepared in the same biological matrix as the clinical samples or in a suitable surrogate biological matrix.
- If needed, stability of the analyte in the presence of co-medication or excipients should also been tested.
- At least three replicates at low and high QC levels should be tested for each stability experiment.
- Enough volume should be prepared for each replicate at each QC level to enable analysis at time zero and after the storage conditions at the different time points of the stability experiments.
- Fresh QC samples and calibration samples should also be prepared for each stability batches and compared to the stability QC samples. If storage needs to be used before experiment, stability of the analyte and IS under the required storage conditions should have already been proven and reason for not using fresh QC samples and calibrators should be given.

**Acceptance criteria and corrective measures**

*Extrapolation and bracketing*

- For small molecules, it is acceptable to extrapolate the stability of the analyte and IS in buffer or in biological matrix from one temperature to a lower one. For instance, if stability is proven at -20°C, then the analyte is also considered stable at -80°C over the tested time period.
- For biological drugs (peptides, proteins and antibody-like drugs), it is acceptable to use a bracketing approach. For instance, if analyte is proven to be stable between +4°C and -80°C, then it is also considered stable at -20°C over the same time period.

*Acceptance criteria*

Mean accuracy at each storage condition for each concentration level should be within ± 15% of nominal concentration.

*Action if failing criteria*

- If stability cannot be proven under the tested storage conditions, then storage conditions must be revised (either shorter storage time or lower storage temperature).
- If no storage condition is proven to be appropriate, solutions must always be prepared fresh from reference substances.

- If stability of analyte in biological matrix fails to be proven over a long time period, care should be taken within the study to analyze the study samples as fast as possible.

**Documentation and stability statement**

*Documentation*

If these investigations are carried out as part of the method validation, they must be reported in the validation report. In any case, a stability statement should be included in the validation report.

*Stability statement*

As a rule, the stability statement applies only to the respective biological matrix for which the stability study was carried out. If a corresponding temporal trend is foreseeable in the storage conditions carried out, the storage conditions must be changed in good time (ideally before trial sample storage). If stability is not guaranteed when the study samples are stored at -20 ° C, the samples should normally be stored at <-70 ° C.

## 4. Related requirements for analytical runs

### 4.1. Calibration curve

**Key parameters**

- All analytical runs should include calibration standards at zero level and at least six non-zero levels, including LLOQ and ULOQ.
- Calibration standards should be prepared in the same biological matrix as the clinical samples.
- Calibration range should cover the expected concentration range of clinical samples.
- Same curve model, weighting parameters, and goodness of fit as those used for method validation should be used.

**Acceptance criteria**

- ✓ Analytical runs should have at least 75% of non-zero calibration standards on at least six different concentration levels into the ± 15% accuracy limits, except at the LLOQ where it should be within ±20%.
- ✓ If calibration samples were prepared in duplicates, at least 50% of each concentration level should meet the criterion. If criterion is not met, concentration level is excluded and calibration curve parameters are recalculated.
- ✓ If the rejected calibration level is the LLOQ, the new LLOQ of the analytical run is the next lowest accepted concentration level (accuracy and precision with ±15% acceptance criteria).
- ✓ If the rejected calibration level is the ULOQ, the new ULOQ of this analytical batch is the previous highest concentration level. The revised calibration range should cover at least three QC levels and clinical samples that are above the new ULOQ should be reanalyzed with dilution.
- ✓ CAL 0 should be free of interferences at the analyte retention time.
- ✓ Analyte response at LLOQ should be at least five times the analyte response in CAL 0.

### 4.2. QC samples

**Key parameters**

- All analytical runs should include QC samples on at least three concentration levels, as for validation (low QC ≤ 3x LLOQ, middle QC = 30-50% calibration range, and high QC ≥ 75% ULOQ).
- QC samples should be prepared in the same biological matrix as clinical samples.
- Number of QC samples should represent at least 5% of the number of clinical samples to be analyzed in one run. In any case, a minimum of six QC samples, i.e. three QC levels in duplicates, should be prepared.
- QC samples are used to make sure that the accuracy and precision of the measurement is maintained throughout the run. They should thus be analyzed throughout the batch in alternation with clinical samples.
- If study samples concentrations are clustered in an unexpected narrow range, additional QC samples should be performed to cover this narrow range. If these additional QC samples are not bracketed by validated QC samples, precision and accuracy should be assessed. If additional QC levels are validated, already analyzed samples do not require re-analysis. At least two QC levels should fall within the concentration range of study samples.

**Acceptance criteria**

- ✓ Analytical runs should have at least 67% of QC samples with within-run accuracy into the ±15% limits and within-run precision below 15% CV.
- ✓ At least 50% of each QC level should meet the criterion. If criterion is not met, concentration level is excluded.
- ✓ At least three QC level should be accepted.
- ✓ Over the complete analytical study, between-run accuracy should fall into the ±15% limits and between-run precision should be below 15% CV.

### 4.3. Specificity and carry-over

**Key parameters**

- BV should be used in each batch for selectivity confirmation.
- Carry-over, if meaningful, should be monitored using eluent samples after ULOQ standards or on the washed MALDI metal target after analysis.

**Acceptance criteria**

- ✓ BV should be free of interferences at the analyte and IS retention times. IS signal in BV samples should be lower than 5% the mean IS signal of QC samples and CALs, and analyte signal in BV should be lower than 20% of the lowest analyte signal of the lowest CAL.
- ✓ Carry-over should not exceed 20% of lowest signal of the lowest CALs and 5% of lowest IS signal in QC samples and calibrators.

### 4.4. Global considerations for rejected analytical runs

- If the listed criteria (points marked as ✓) are not fulfilled, the analytical run must be rejected and a new analytical run needs to be prepared for all study samples included in the failed run.
- QC data from all passed and rejected runs should be reported but only QC data from passed runs should be used for between-run precision and accuracy.

- For multiple analyte assays, there should be one calibration curve and one QC sample set for each analyte. One failing analyte does not make all the other valid analyte rejected.

### 4.5. Incurred sample reanalysis

**General considerations**

*Aim*

Incurred sample reanalysis (ISR) batch is intended to verify the reliability of the bioanalytical assay. It is conducted by repeating the analysis of a subset of samples from a given study in separate (i.e., different to the original) runs on different days using the same bioanalytical method.

*Key parameters*

- Incurred samples should not be pooled.
- Incurred samples should represent 10% of the total number of clinical samples for study with up to 1000 samples and 5% of the remaining number should be added.
- ISR samples should be selected to be representative of the complete study and should be selected around the $c_{max}$ region and in the elimination phase.
- Samples selected for ISR batch should have an original calculated concentration above 3*LLOQ and below 80% ULOQ to avoid any ISR results below LLOQ and above ULOQ.
- If original samples were processed in replicates, ISR samples should also be processed in replicates.
- ISR batch should be done as soon as possible in the clinical sample analysis.

**Acceptance criteria and corrective measures**

*Acceptance criteria*

- Difference between original and repeated value is calculated as difference to the mean:

$$\%\ diff = \frac{(repeated\ value - original\ value)}{mean\ value} \times 100$$

- If some repeated values are below LLOQ or above ULOQ, the samples should be excluded from the ISR calculations.
- At least 67% of repeated values should be within ±20% to the mean.

*Action if failing criteria*

Reasons for failing criteria should be investigated and method should be revised. If the next ISR batch is also rejected, study sample analysis should be stopped until method revision and validation of an ISR batch.

### 4.6. Cross-validation (data bridging) of desorption/ionization-based assays

**General considerations**

- As validated quantification DI assays are still not very common, it might be necessary for each new assay to perform a cross-validation (data bridging) with another validated gold-standard quantification method (e.g. LC-MS/MS or ligand-binding assay) in order to bring an additional level of confidence.

  However, it must be noted that the ICH guidelines [3] suggest that a standalone newly developed and validated assay using new analytical instrumentation might not require cross-validation. But if such an assay is destined to be used in parallel to another bioanalytical assay using a different analytical platform or to replace this latter, it should be cross-validated to make sure that results will be comparable between the two approaches.

- In any case, if the full validation is not possible for DI assays, the method must be validated at least using cross-validation of results with LC-MS/MS.
- Cross-validation of results using samples dosed *in vivo* or *in vitro* might also be useful during method development to make sure that the developed DI assay yields similar results to LC-MS/MS (for instance) before starting any validation.

*Key parameters*

- Cross-validation is conducted as an ISR batch.
- At least 20 samples should be analyzed for the cross-validation of DI-based assay with another established analytical approach (e.g. LC-MS/MS).
- Samples selected for cross-validation should have an original calculated concentration above 3 x LLOQ and below 80% ULOQ to avoid any results below LLOQ and above ULOQ.

**Acceptance criteria and corrective measures**

*Acceptance criteria*

- Difference between original and repeated value is calculated as difference to the mean:

$$\%\ diff = \frac{(repeated\ value - original\ value)}{mean\ value} \times 100$$

- If some bridged values are below LLOQ or above ULOQ, the samples should be excluded from the calculations.
- At least 67% of the bridged values should be within ±20% to the mean.

*Action if failing criteria*

- Reasons for failing criteria should be investigated and method should be revised. If the next cross-validation batch is also rejected, study sample analysis using the DI-based assay should be stopped until method revision and validation of a cross-validation batch.
- If a reproducible difference to the mean is observed between the bridged values (precision of the difference to the mean ≤ 15% CV), a corrective coefficient might be applied to the quantification results and the DI-based assay used for biological sample analyses.

## II. Specific sample preparation strategies for bioanalytical method validation

1. Desorption/ionization methods for drug quantification in liquid biological matrices

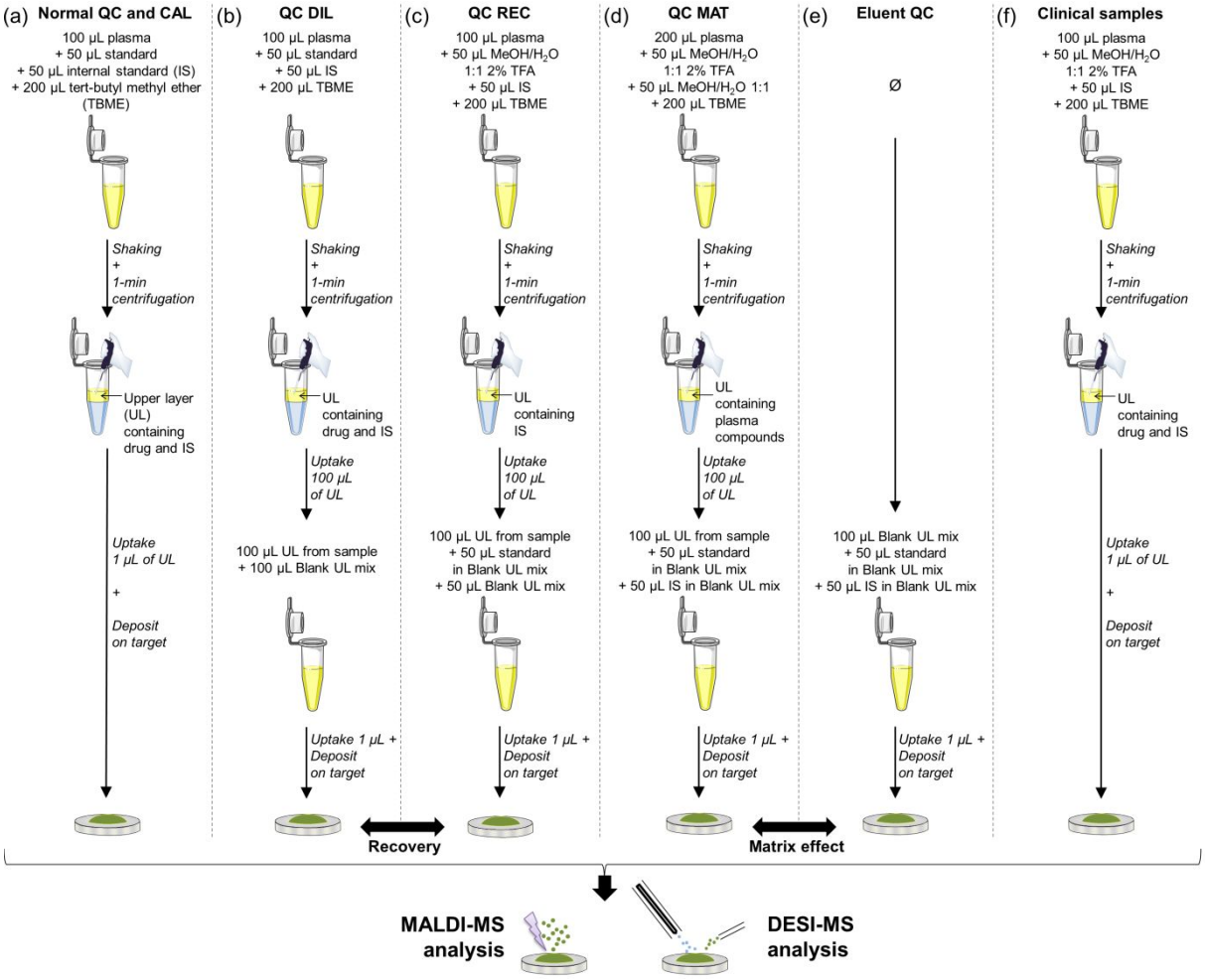


Figure S1. Example workflow for the preparation of the quality control (QC), calibration standard (CAL), and clinical samples during validation and analytical batches of a desorption/ionization – mass spectrometry (DI-MS) assay for drug quantification in liquid biological matrix using liquid-liquid extraction (LLE) followed by direct analysis. (a) Preparation of CAL samples for all batches and normal QC samples for accuracy and precision batches, and for analytical batches. (b) Preparation of QC DIL (diluted QC) samples for recovery experiments. (c) Preparation of 100%-QCs (QC REC) samples for recovery experiments (corresponding to the hypothesis of total extraction of the drug using the LLE process). (d) Preparation of QC MAT (QC matrix) samples for the biological matrix effect experiments. (e) Preparation of the Eluent-QC for the biological matrix effect experiments. (f) Preparation of the clinical samples for the analytical batches. Adapted with permission from ref.[4]. Copyright 2020, MDPI.

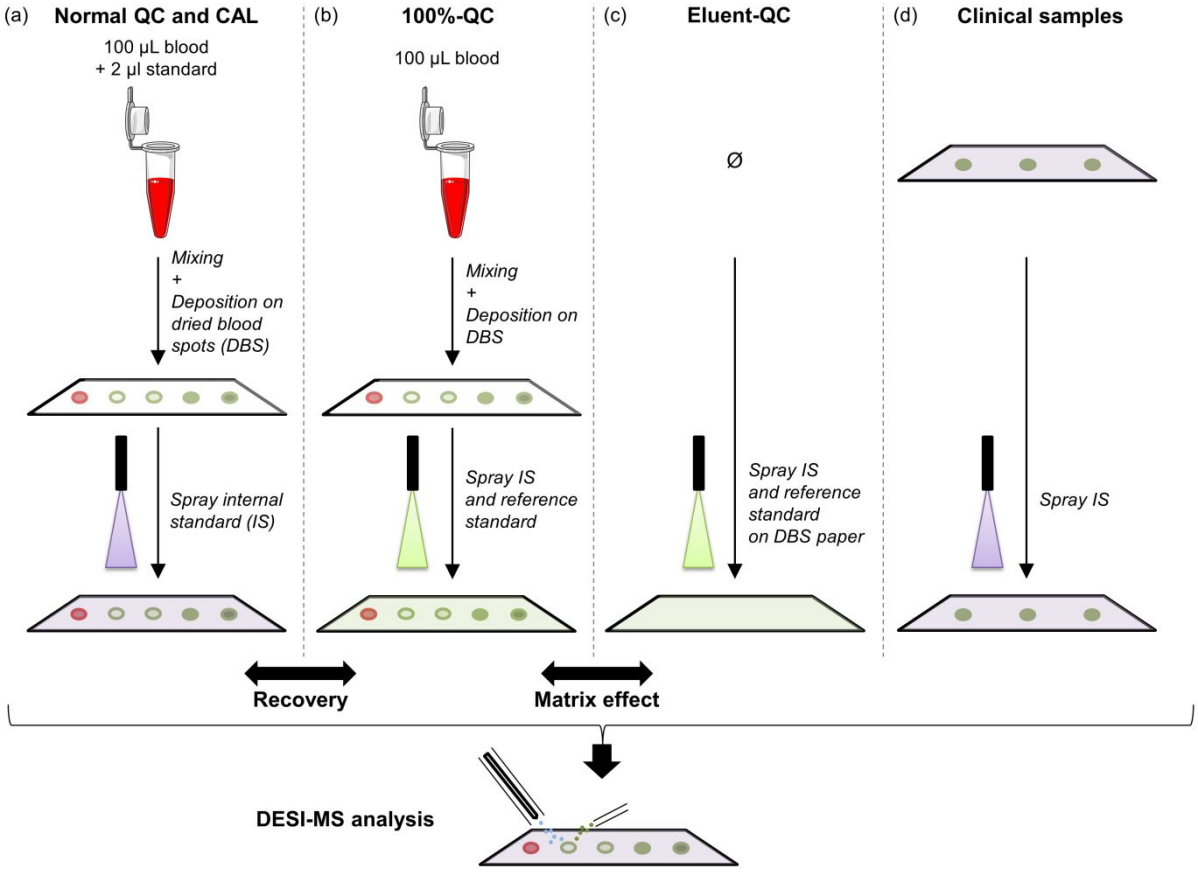


Figure S2. Example workflow for the preparation of the quality control (QC), calibration standard (CAL), and clinical samples during validation and analytical batches of a desorption electrospray ionization (DESI)– mass spectrometry (MS) assay for drug quantification in dried blood spots (DBS). (a) Preparation of CAL samples for all batches and normal QC samples for accuracy and precision batches, and for analytical batches. (b) Preparation of 100%-QC samples for recovery experiments (corresponding to the hypothesis of total extraction of the drug during the IS deposition). (c) Preparation of the Eluent-QC for the biological matrix effect experiments. (d) Preparation of the clinical samples for the analytical batches. Red spots are blank blood and green spots are blood containing drug.

3. Desorption/ionization methods for drug quantification in tissue sections

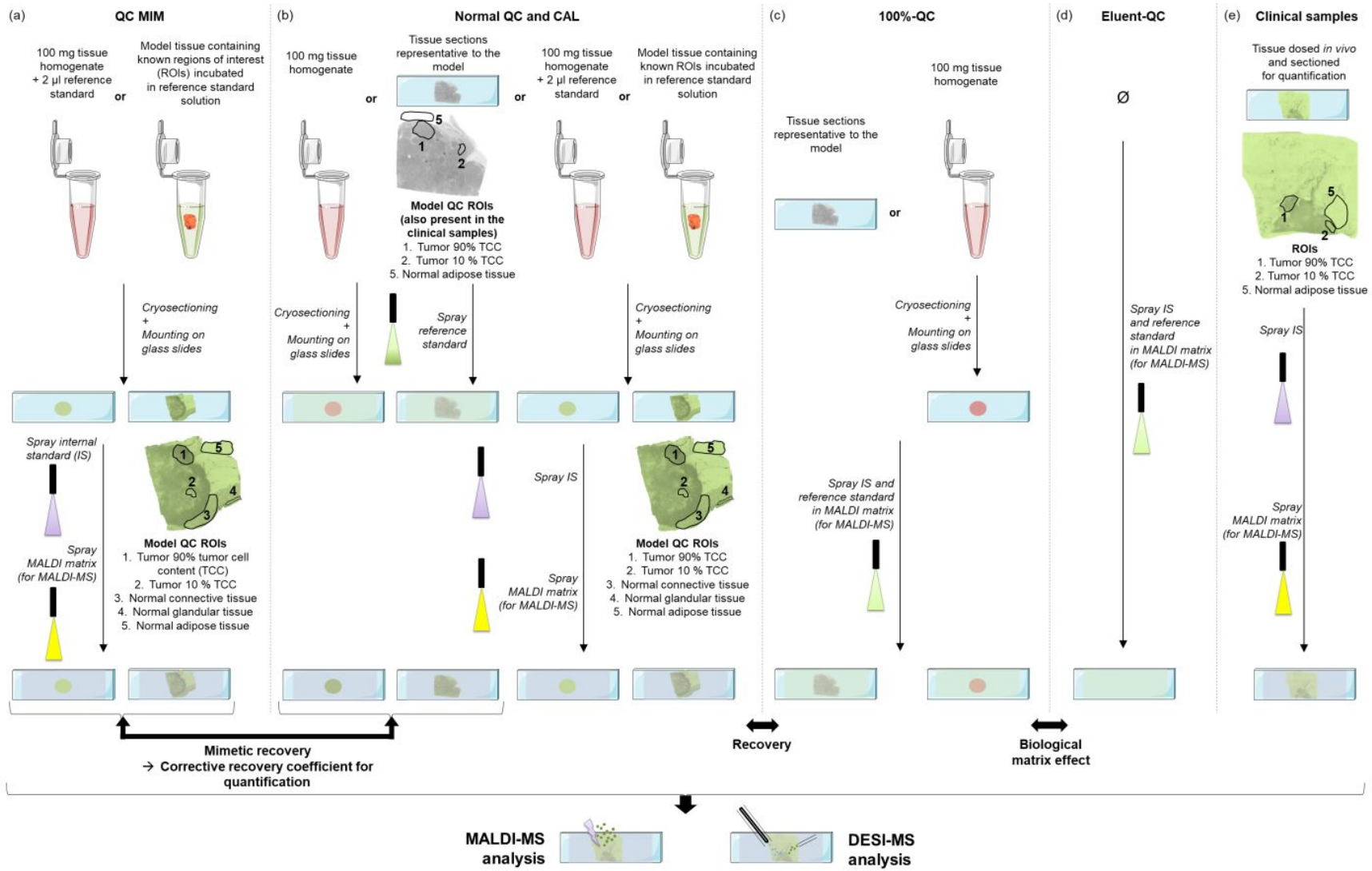


Figure S3. Example workflow for the preparation of the quality control (QC), calibration standard (CAL), and clinical samples during validation and analytical batches of a desorption/ionization – mass spectrometry (DI-MS) assay for drug quantification in tissues. (a) Preparation of tissues dosed *in vitro* for the QC MIM series aiming to represent a realistic model to mimic tissues dosed *in vivo*. (b) Preparation of CAL samples for all batches and normal QC samples for accuracy and precision batches, and for analytical batches. (c) Preparation of 100%-QC samples for classical recovery experiments (corresponding to the hypothesis of total extraction of the drug during the IS deposition). (d) Preparation of the Eluent-QC for the biological matrix effect experiments. (d) Preparation of the clinical samples for the analytical batches.

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