Draft Guideline on Bioanalytical Method (Ligand Binding Assay) Validation in Pharmaceutical Development

(24 January, 2014, MHLW, Japan)

Table of Contents

1. Introduction
2. Scope
3. Reference Standard
4. Analytical Method Validation
4.1. Full validation
    4.1.1. Specificity
    4.1.2. Selectivity
    4.1.3. Calibration curve
    4.1.4. Accuracy and precision
    4.1.5. Dilutional linearity
    4.1.6. Stability
4.2. Partial validation
4.3. Cross validation
5. Analysis of Study Samples
5.1. Calibration curve
5.2. QC samples
5.3. Incurred samples reanalysis (ISR)
6. Points to note
6.1. Calibration range
6.2. Reanalysis
6.3. Carry-over
6.4. Cross-talk
6.5. Critical reagent
6.6. Interfering substances
7. Documentation and Archives
Glossary

1. Introduction

In the development of medicinal products, bioanalytical methods are used in clinical and non-clinical pharmacokinetic studies (including toxicokinetic studies) to evaluate the efficacy and safety of drugs and their metabolites. Drug concentrations determined in biological samples are used for the assessment of characteristics such as in vivo pharmacokinetics (absorption, distribution, metabolism, and excretion), bioavailability, bioequivalence, and drug-drug interaction.

It is important that these bioanalytical methods are well characterized throughout the analytical procedures to establish their validity and reliability.

This guideline serves as a general guidance recommended for the validation of bioanalytical methods based on ligand-binding assays to ensure adequate reliability. It also provides a framework for analyses of study samples by using validated methods to evaluate study results supporting applications for drug marketing authorization.

Flexible adjustment and modification can be applied in case of using the specific type analytical method or depending on the intended use of the result of analysis, such as the use of prospectively defined appropriate criteria, based on scientific rationale.

2. Scope

This guideline is applicable to the validation of analytical methods based on ligand binding assays (LBAs) to measure concentrations of drugs in biological samples obtained in toxicokinetic studies and clinical trials, as well as to the analyses of study samples using such methods. The information in this guideline generally applies to the quantification of peptides and proteins as well as low-molecular-weight drugs that are analyzed by LBAs. A typical example of LBA is immunological assay based on antigen-antibody reaction, such as **enzyme immunoassay (EIA).**

This guideline is not mandatory for analytical methods used in non-clinical studies that are beyond the scope of "Ministerial Ordinance Concerning the Standards for the Conduct of Non-clinical Studies on the Safety of Drugs (Ministry of Health and Welfare ordinance No. 21, dated March 26, 1997)," but could be used as a reference for conducting a method validation for a non GxP bioanalysis.

3. Reference Standard

A reference standard serves as the scale in quantifying an analyte, and is mainly used to prepare calibration standards and quality control (QC) samples, which are relevant blank matrix spiked with a known concentration of the analyte of interest. The quality of the reference material is critical, as the quality affect measurement data. A certificate of analysis or an alternative statement that provides information on lot number, content (amount, purity, or potency), storage conditions, and expiration date or re-test date should accompany the standard. As a reference standard, it is necessary to show its

39 authenticated source and its characteristics should be well-established.

40 **4. Analytical Method Validation**

41 An analytical method validation should be performed at every relevant facility when
42 establishing a bioanalytical method for quantification of a drug or its metabolite(s).

43 4.1. Full validation

44 A full validation should be performed when establishing a new bioanalytical method
45 for quantification of an analyte/analytes. A full validation is also required when
46 implementing an analytical method that is disclosed in literature or commercialized as a
47 kit product.
48 The objective of a full validation is to demonstrate the assay performance of the
49 method, e.g., specificity, selectivity, calibration curve, accuracy, precision, dilutional
50 linearity, and stability. Generally, a full validation should be performed for each species
51 and matrix (mainly plasma or serum) to be analyzed.
52 The matrix used in analytical validation should be as close as possible to the target
53 study samples, including anticoagulants and additives. When an analytical method is to
54 be established for a matrix of limited availability (rare matrix, e.g., tissue, cerebrospinal
55 fluid, bile), a sufficient amount of matrix cannot be obtained from sufficient number of
56 sources (subjects or animals). In such a case, a surrogate matrix may be used to prepare
57 calibration standards and QC samples. However, the use of a surrogate matrix should be
58 rigorously justified in the course of establishing the analytical method.
59 In an LBA validation, full validation should be conducted using samples diluted at a
60 factor of minimum required dilution (MRD), which has been determined in the course of
61 method development. In a plate-based LBA, assay should generally be performed in at
62 least duplicate (2 wells) per processed sample. The sample concentration should be
63 calculated from a mean of response variable of each well, or from taking an average of
64 concentrations which are determined from response variables of each well.

65 4.1.1. Specificity

66 Specificity is the ability of an analytical method to detect and differentiate the analyte
67 from other substances such as its related substances. For LBA, it is important that
68 binding reagent specifically binds to the target analyte but does not cross-react with
69 coexisting substances that are structurally similar to the analyte. If similar substances are
70 expected to be present in biological samples of interest, the extent of the impact of such
71 substances on the analysis of analyte should be evaluated. It is acceptable that specificity
72 is evaluated in the course of method development or after completing a method
73 validation.
74 Specificity is evaluated using blank sample (matrix sample without analyte addition),
75 blank sample spiked with similar substance at an expected concentration, and near-low-

and near-high-level QC samples spiked with similar substance at an expected concentration.

Assay result for the blank sample should be below the lower limit of quantification (LLOQ), and accuracy in the measurements of the QC samples spiked with similar substance should be within ±20% of the theoretical concentration (or within ±25% of the theoretical concentration at the LLOQ and upper limits of quantification [ULOQ]).

4.1.2. Selectivity

Selectivity is the ability of an analytical method to measure and differentiate the analyte in the presence of other components in samples.

Selectivity is evaluated using blank samples obtained from at least 10 individual sources and near-LLOQ QC samples prepared from individual blank samples. In case of a matrix with limited availability, it is acceptable to use matrix samples obtained from less than 10 sources.

Assay results for at least 80% of the blank samples should be below the LLOQ, and accuracy of the measurements of at least 80% of the near-LLOQ QC samples should be within ±20% of the theoretical concentration (or ±25% at the LLOQ).

4.1.3. Calibration curve

A calibration curve demonstrates the relationship between a theoretical concentration and a response variable for an analyte.

A calibration curve should be prepared using the same matrix as the intended study samples, whenever possible, by spiking the blank matrix with known concentrations of the analyte. A calibration curve should be generated with at least 6 concentration levels of calibration standards, including LLOQ and ULOQ samples, and a blank sample. Anchor point samples at concentrations below LLOQ and above ULOQ may also be used to improve curve fitting. A 4- or 5-parameter logistic model is generally used for the regression equation of calibration curve. The validation report should include the regression equation and weighting conditions used.

The accuracy of back-calculated concentration of each calibration standard should be within ±25% deviation of the theoretical concentrations at the LLOQ and ULOQ, or ±20% deviation at all other levels. No accuracy criteria are specified for anchor points. At least 75% of the calibration standards excluding anchor points, and a minimum of 6 levels, including the LLOQ and ULOQ, should meet the above criteria.

4.1.4. Accuracy and precision

Accuracy of an analytical method describes the degree of closeness between analyte

concentration determined by the method and its theoretical concentration. Precision of an analytical method describes variation between individual concentrations determined in repeated measurements.

Accuracy and precision are assessed by performing analysis with QC samples, i.e., samples spiked with known amounts of the analyte. QC samples are processed in the same manner as in the analysis of study samples. In the validation, QC samples with a minimum of 5 different concentrations (LLOQ, low-, mid-, high-levels, and ULOQ) within the calibration range are prepared. The low-level should be within 3 times the LLOQ, the mid-level is around the midpoint on the calibration curve, and the high-level should be at least one-third of the ULOQ of the calibration curve. Within-run and between-run accuracy and precision should be evaluated by repeating the analysis run at least 6 times.

The mean accuracy at each concentration level should be within ±20% deviation of the theoretical concentration, except at the LLOQ and ULOQ, where it should be within ±25%. Precision of concentrations determined at each level should not exceed 20%, except at the LLOQ and ULOQ, where it should not exceed 25%. Furthermore, a sum of absolute accuracy and precision (total error) at each level should not exceed 30%, except at the LLOQ and ULOQ, where it should not exceed 40%.

4.1.5. Dilutional linearity

Dilutional linearity is assessed to confirm that the method can appropriately analyze samples at concentrations exceeding the ULOQ without influence of a hook effect or prozone effect and that these measurements are not affected by dilution within the calibration range. Dilutional linearity is evaluated by analyzing a QC sample exceeding the ULOQ and its serially-diluted samples at multiple concentrations. The absence or presence of response reduction (hook effect or prozone effect) is checked in the analyzed samples, and measures should be taken to eliminate response reduction in study sample analysis, when applicable. Accuracy and precision in the measurements corrected for the dilution factor should be within ±20% deviation of the theoretical concentration and not more than 20%, respectively.

4.1.6 Stability

Analyte stability should be evaluated to ensure that the concentration is not affected by the samples through each step of the process from the sample collection to the analysis. The stability of the samples should be assessed under conditions that are as close to the actual circumstances, e.g. sample storage and sample analysis as much as possible. Careful consideration should be given to the solvent or matrix type, container materials, and storage conditions used in the stability-determination process.

Validation studies should determine analyte stability after freeze and thaw cycles, after

short-term (at room temperature, on ice, or under refrigeration) and long-term storage. All stability experiments should be performed on samples that have been stored for a time that is longer than the actual storage period.

Stability of the analyte in the stock and working solution is evaluated using solutions at the highest and lowest concentration levels in the actual storage.

Stability of the analyte in the studied matrix is evaluated using low- and high-level QC samples. The QC samples should be prepared using a matrix that is as close as possible to the actual study samples, including anticoagulant and additives. Stability is evaluated by at least 3 replicates per concentration level with QC samples before and after storage. The mean accuracy in the measurements at each level should be within ±20% deviation of the theoretical concentration, in principle. If other criteria are more appropriate for the evaluation of a specific analyte, they could be used.

4.2. Partial validation

Partial validation may be performed when minor changes are made to an analytical method that has already been fully validated. The items in a partial validation are determined according to the extent and nature of the changes made to the method.

Typical bioanalytical method changes subject to a partial validation are as follows: analytical method transfers between laboratories, changes in analytical instruments, changes of the critical reagent lot, changes in calibration range, changes in MRD, changes in anticoagulant, changes in analytical conditions, changes in sample storage conditions, confirmation of impact by concomitant drugs, and use of rare matrices.

Acceptance criteria used in partial validation should be the same as those employed in the full validation in principle.

4.3. Cross validation

Cross validation is primarily conducted when data are generated in multiple laboratories within a study or when comparing analytical methods used in different studies, after a full or partial validation. The same set of QC samples spiked with the analyte or the same set of study samples is analyzed, and the mean accuracy at each concentration level of QC samples or the assay variability in the measurements of study samples is evaluated.

In the cross validation among two or more laboratories within a study, the mean accuracy of QC samples (low-, mid-, and high-levels) evaluated by at least 3 replicates at each level, should be within ±30% deviation of the theoretical concentration, in principle, considering intra- and inter-laboratory precision. When using a set of study samples, the assay variability should be within ±30% for at least two-thirds of the samples.

In the cross validation between different analytical methods based on different assay principles, both validation procedure and acceptance criteria (i.e., mean accuracy or assay

191 variability) should be separately defined based on scientific judgment according to the
192 type of the analytical methods.
193

194 **5. Analysis of Study Samples**

195 Study samples are biological specimens that are obtained from toxicokinetic studies
196 and clinical trials Analysis of study samples should be carried out using a fully validated
197 analytical method. In the analysis, study samples should be handled under conditions that
198 are validated for adequate stability, and analyzed within a confirmed stability period,
199 along with a blank sample, calibration standards at a minimum of 6 concentration levels,
200 and QC samples at a minimum of 3 concentration levels. In a plate-based LBA, assay
201 should generally be performed in at least duplicate (2 wells) per processed sample to
202 calculate a sample concentration from a mean of response variable of each well, or from
203 taking an average of concentrations which are determined from response variables of
204 each well.
205 Validity of the analytical method during study sample analysis should be evaluated in
206 each analytical run by using the calibration curve and QC samples. In plate-based assays,
207 each plate represents a single analytical run. In studies that serve pharmacokinetic data as
208 a primary endpoint, reproducibility of the analytical method should be confirmed for
209 each representative study per matrix by performing incurred sample reanalysis (ISR:
210 reanalysis of incurred samples in a separate analytical run on separate days to determine
211 whether the original analytical results are reproducible).
212

213 5.1. Calibration curve

214 A calibration curve is used to determine the concentration of the analyte of interest in
215 study samples. A calibration curve used in study sample analysis should be generated for
216 each analytical run by using the validated analytical method. The same model as in the
217 bioanalytical method validation should be used for the regression equation and weighting
218 conditions of the calibration curve.
219 The accuracy of back-calculated concentrations of calibration standards at each level
220 should be within ±25% deviation of the theoretical concentration at the LLOQ and
221 ULOQ, or ±20% deviation at all other levels. No accuracy criteria are specified for
222 anchor points. At least 75% of the calibration standards excluding anchor points, with a
223 minimum of 6 levels, should meet the above criteria.
224 If the calibration standard at the LLOQ or ULOQ did not meet the criteria in study
225 sample analysis, the next lowest/highest-level calibration standard may be used as the
226 LLOQ or ULOQ of the calibration curve. Even though narrowed, the modified
227 calibration range should still cover at least 3 different QC sample levels (low-, mid-, and
228 high-levels).
229

230 5.2. QC samples

231 QC samples are analyzed to assess the validity of the analytical method used for
232 calibration curve and study sample analysis.
233 QC samples with a minimum of 3 different concentration levels (low-, mid-, and
234 high-levels) within the calibration range are analyzed in each analytical run. Usually, the
235 low-level is within 3 times the LLOQ, the mid-level is in the midrange of the calibration
236 curve, and the high-level needs to be more than one-third of the ULOQ of the calibration
237 curve. QC samples are processed in the same manner as study samples. The analysis
238 requires 2 QC samples at each QC level or at least 5% of the total number of study
239 samples in the analytical run, whichever is the greater.
240 The accuracy in measurement of QC samples should be within ±20% deviation of the
241 theoretical concentrations. At least two-thirds of the QC samples and at least 50% at each
242 concentration level should meet the above criterion.
243

244 5.3. ISR (Incurred sample reanalysis)

245 In bioanalysis, it can happen that the results of analyses of study samples are not
246 reproducible, even when the method validation is successfully conducted and the validity
247 of at each analytical run is confirmed by calibration standards and QC samples. Such
248 failures can be attributed to various factors, including inhomogeneity of study samples,
249 contamination and other operational errors, and interference of biological components
250 unique to the study samples or of unknown metabolites. ISR refers to reanalysis of
251 incurred samples in separate analytical runs on different days to check whether the
252 original analytical results are reproducible. Confirmation of the reproducibility by ISR
253 improves the reliability of the analytical data. In addition, a failure to demonstrate the
254 reproducibility of the original data in ISR can trigger a cause investigation and remedial
255 measures for the analytical method.
256 Usually, ISR is performed for representative studies selected for each matrix in studies
257 that use pharmacokinetic data as the primary endpoint. For instance, ISR should be
258 conducted in the following situations: non-clinical toxicokinetic studies for individual
259 species, representative clinical pharmacokinetic studies in healthy volunteers and
260 renal/hepatic impaired patients, as well as bioequivalence studies. For non-clinical
261 studies, ISR may be performed with samples obtained in a preliminary non-clinical study,
262 if the study design is similar to the relevant toxicokinetic study in terms of sampling
263 conditions.
264 ISR should be performed with samples from as many subjects or animals as possible,
265 including those near the maximum blood concentration (Cmax) and the elimination
266 phase, within a time window that ensures the analyte stability. As a guide, approximately
267 10% of the samples should be reanalyzed in cases where the total number of study
268 samples is less than 1000 and approximately 5% of the number of samples exceeding
269 1000.

270 The results of ISR are evaluated using assay variability. Assay variability can be
271 calculated as the difference between the concentration obtained by ISR and that in the
272 original analysis divided by their mean and multiplied by 100. The assay variability
273 should be within ±30% for at least two-thirds of the samples analyzed in ISR. If the ISR
274 data failed to meet the above criteria, cause investigation should be conducted for the
275 analytical method and necessary measures should be taken by considering the potential
276 impact on study sample analysis.
277 It should be noted that ISR is performed to monitor assay variability. The original data
278 should never be discarded or replaced with the reanalysis data even if the assay
279 variability exceeds ±30% in a specific sample.
280

281 **6. Points to note**

282 6.1. Calibration range

283 In LBAs, calibration range is largely dependent on the characteristics of the binding
284 reagents and it may be difficult to arbitrarily determine the range. In such cases, care
285 must be exercised to appropriately select the range of dilutional linearity.
286 In case the calibration range is changed, partial validation should be performed.
287 However, it is not necessary to reanalyze the study samples that have been quantified
288 prior to the change in the calibration range, levels, or number of QC samples.
289

290 6.2. Reanalysis

291 Possible reasons and procedures for reanalysis, as well as criteria for handling of
292 concentration data should be predefined in the protocol or standard operating procedure
293 (SOP).
294 Examples of reasons for reanalysis are as follows: calibration curve or QC samples
295 failed to meet the criteria for validity of the analytical run; the obtained concentration
296 exceeded the ULOQ of the calibration curve or fell below the LLOQ due to excess
297 dilution; the analyte of interest was detected in pre-dose or placebo samples; improper
298 analytical operation or malfunction of analytical instrument; and causal investigation on
299 abnormal values.
300 Reanalysis of study samples for a pharmacokinetic reason should be avoided,
301 whenever possible. Particularly in bioequivalence studies, it is not acceptable to
302 reanalyze study samples and replace the concentration data only because the initial data
303 were pharmacokinetically questionable. However, reanalysis of specific study samples
304 are acceptable when, for instance, the initial analysis yielded an unexpected or
305 anomalous result that may affect the safety of subject in a clinical trial.
306 In any case, when reanalysis is performed, the analytical report should provide

307 information of the reanalyzed samples, the reason for reanalysis, the data obtained in the
308 initial analysis, if any, the data obtained in the reanalysis, and the final accepted values
309 and the reason and method of selection.
310

311 ### 6.3. Carry-over

312 Carry-over is an alteration of a measured concentration due to residual analyte in the
313 analytical equipment.
314 Carry-over is not a problem for analyses performed in plates and tubes, while carry-over
315 should be taken into account in analyses that use a single flow cell, flow path, and/or
316 autosampler.
317 If carry-over is inevitable, the impact of carry-over needs to be evaluated, and
318 appropriate measures should be taken to avoid any impact on the actual study sample
319 analysis. Should there be any concern that carry-over may affect the quantification of
320 analyte in study samples, it should be evaluated during the actual study sample analysis
321 to assess the impact on the concentration data.
322

323 ### 6.4. Cross-talk

324 Cross-talk is an alteration of a measured concentration due to leak of fluorescent or
325 luminescent light to adjacent wells in plate-based assay.
326 If cross-talk is inevitable, the impact of cross-talk needs to be examined, and
327 appropriate measures should be taken to avoid any impact on the actual study sample
328 analysis. Should there be any concern that cross-talk may affect the quantification of
329 analyte in study samples, this should be evaluated during the actual study sample analysis
330 to assess the impact on the concentration data.
331

332 ### 6.5. Critical reagent

333 Critical reagents are usually binding reagents (labeled or unlabeled antibodies) that
334 have a direct impact on the results of ligand-binding-based bioanalytical methods.
335 A critical reagent should be selected by considering the specificity for the analyte and
336 stored under conditions that ensure consistent quality. The quality of critical reagent
337 should be appropriately maintained throughout the period of use in analytical method
338 validation and study sample analyses. Partial validation is required when the critical
339 reagent lot is changed.
340

341 ### 6.6. Interfering substances

342 Interfering substances are soluble ligands to drugs and anti-drug antibodies that may

343 affect the concentration data in study sample analysis.
344 If interfering substances are potentially present in study samples, it is advisable to
345 examine the impact of interfering substances on the concentration data.
346

347 **7. Documentation and Archives**

348 In order to ensure adequate reproducibility and reliability of bioanalysis, results
349 obtained in analytical method validations and study sample analyses should be
350 documented in a validation report and a study sample analysis report as described below.
351 The reports should be stored along with relevant records and raw data in an appropriate
352 manner.
353 All relevant records and raw data should be kept, including those obtained in rejected
354 analytical runs, specifically record of reference materials, blank matrices, and critical
355 reagents (receipt/release, use, and storage), record of samples (receipt/release,
356 preparation, and storage), record of analyses, record of instrument (calibration and
357 settings), record of deviations, record of communications, and raw data such as analytical
358 data.

359

360 **Validation report**

361

362 ● Summary of the validation
363 ● Information on the reference standards
364 ● Information on the blank matrices
365 ● Information on the critical reagents
366 ● Analytical method (description including MRD)
367 ● Validated parameters and the acceptance criteria
368 ● Validation results and discussion
369 ● Rejected runs together with the reason for rejection
370 ● Information on reanalysis
371 ● Deviations from the protocol and/or SOP, along with the impact on study results
372 ● Information on reference study, protocol, and literature
373

374 **Study sample analysis report**

375

376 ● Summary of the study sample analysis
377 ● Information on the reference standards
378 ● Information on the blank matrices
379 ● Information on receipt and storage of study samples
380 ● Information on the critical reagents
381 ● Analytical method

382 - Parameters, acceptance criteria, and results of the validity evaluation
383 - Results and discussion of study sample analysis
384 - Rejected runs together with the reason for rejection
385 - Information on reanalysis
386 - Deviations from the protocol and/or SOP, along with impact on study results
387 - Information on reference study, protocol, and literature
388
389
390
391

392 **Glossary**

393

394 **Accuracy:** The degree of closeness of a concentration determined by the method to the
395 theoretical concentration. Accuracy is expressed as a percentage relative to the
396 theoretical concentration.

397 Accuracy (%) = (Measured concentration/Theoretical concentration) × 100.

398 **Analysis:** A series of analytical procedures from sample dilution to measurement on an
399 analytical instrument.

400 **Analyte:** A series of analytical procedures from sample processing to measurement on an
401 analytical instrument.

402 **Analytical run:** A set of samples comprising calibration standards, QC samples, and
403 study samples. Usually, a set of samples is prepared without interruption in time by a
404 single analyst with the same reagents under the same conditions, and subsequently
405 analyzed in a single plate as a single analytical run.

406 **Anchor point:** Samples at a concentration below the LLOQ or above the ULOQ and
407 analyzed concurrently with calibration standards to improve curve fitting.

408 **Assay variability:** The degree of difference in concentrations determined for the same
409 sample. The difference is expressed as a percentage relative to the mean of the two.

410 Assay variability (%) = [(Concentration in comparative analysis - Concentration in
411 reference analysis) / Mean of the two] × 100.

412 **Binding reagent:** A reagent used in LBA that directly binds to the analyte.

413 **Blank sample:** A matrix sample that is analyzed without analyte addition.

414 **Calibration curve:** Presentation of the relationship between concentration and
415 response for an analyte. A calibration curve is generated with at least 6 concentration
416 levels of calibration standards, including LLOQ and ULOQ samples, as well as a blank
417 sample. Anchor points may be added outside the calibration range.

418 **Calibration standard:** A sample spiked with the analyte of interest to a known
419 concentration, which is used to generate calibration curves. Calibration standards are
420 used to generate calibration curves, from which the concentrations of the analyte in QC
421 samples and study samples are determined.

422 **Carry over:** An alteration of the measured concentration due to leftover analyte in the
423 analytical instrument used.

424 **Critical reagent:** A critical reagent that has a direct impact on the results of a
425 ligand-binding-based bioanalytical method. Binding reagent is mainly considered as
426 critical reagent.

427 **Cross validation:** A validation conducted when data are generated in multiple
428 laboratories within a study or when comparing analytical methods used in different
429 studies. Cross validation is performed after a full or partial validation.

430 **Cross-reactivity:** Binding of the binding reagent to other substances except for (or
431 other than) the analyte.

432 **Cross-talk:** An alteration of the measured concentration due to leak of fluorescent or
433 luminescent light from adjacent wells in plate-based assay.

434 **Dilutional linearity:** A parameter demonstrating that the method can appropriately
435 analyze samples at a concentration exceeding the ULOQ without influence of hook effect
436 or prozone and that these measurements are not affected by dilution within the calibration
437 range.

438 **Full validation:** A validation that evaluates a full panel of performance parameters, i.e.,
439 specificity, selectivity, calibration curve, accuracy, precision, dilutional linearity, and
440 stability. A full validation is usually performed when establishing a new bioanalytical
441 method.

442 **Hook effect:** Suppression of response in very high concentration levels of analyte.
443 Special care should be taken on the results obtained when the hook effect is observed,
444 because samples at a concentration exceeding the ULOQ may falsely produce results
445 within or below the calibration range. The hook effect often occurs in a LBA that
446 performs liquid-phase reaction of binding reagent with analyte.

447 **Incurred sample:** A study sample that is obtained from a subject or animal that was
448 dosed with an active study drug.

449 **Incurred sample reanalysis (ISR):** Reanalysis of a portion of the incurred samples in a
450 separate analytical run on a different day to determine whether the original analytical
451 results are reproducible.

452 **Interfering substance:** Substance that is present in the matrix and may affect interaction
453 between the binding reagent and the analyte.

454 **Ligand binding assay:** A method to analyze an analyte using a binding reagent that
455 specifically binds to the analyte. Antigen-antibody reaction is utilized in the majority of
456 ligand binding assay. The analyte is detected using a reagent labeled with an enzyme,
457 radioisotope, fluorophore, or luminophore. Reaction is carried out in 96-well microtiter
458 plates, test tubes, disks or others.

459 **Lower limit of quantification (LLOQ):** The lowest concentration of an analyte at
460 which the analyte can be quantified with reliable accuracy and precision.

461 **Matrix:** Whole blood, plasma, serum, urine, or other biological fluid or tissue selected
462 for analysis. A matrix not containing exogenous chemicals (except anticoagulant) and
463 their metabolites is called a blank matrix.

464 **Minimum required dilution (MRD):** A dilution factor of samples (including
465 calibration standards and QC samples) with buffer to analyze samples appropriately.
466 MRD should be identical for all samples. It may not necessarily be the exact minimum
467 dilution where samples can be analyzed. Quality control (QC) sample: A sample spiked
468 with the analyte of interest to a known concentration used to assess the reliability of an
469 analytical method. In analytical runs, QC samples are analyzed to assess the validity of
470 the analytical method used for calibration curve and study sample analysis.

471 **Partial validation:** A validation performed when minor changes are made to an
472 analytical method that has already been fully validated. A set of parameters to be
473 evaluated in a partial validation should be determined according to the extent and nature
474 of the changes made to the method. It can range from as little as accuracy and precision
475 evaluation in a day to a nearly full validation.

476 **Precision:** The degree of closeness between individual concentrations determined in
477 repeated measurements. Precision is expressed as the coefficient of variation (CV) or the
478 relative standard deviation (RSD) in percentage.

479 Precision (%) = (Standard deviation/Mean) × 100.

480 **Prozone:** Suppression of response in very high concentration levels of analyte. This is
481 the same phenomenon as the hook effect.

482 **Quantification range:** The range of concentration of an analyte in which the analyte can
483 be quantified with reliable accuracy and precision. Quantification range of a
484 bioanalytical method is ensured by the range of calibration curve (calibration range) and
485 the dilution integrity.

486 **Reanalysis:** Repetition of a series of analytical procedures from the dilution step on
487 samples that have been analyzed once.

488 **Reference material (Reference standard):** A compound used as the standard in
489 quantifying an analyte; mainly used to prepare calibration standards and QC samples.

490 **Response variable:** A response obtained from the detector of the analytical instrument.
491 In LBAs, response is generally monitored by a spectroscopic technique, which converts
492 the response into an electrical signal, such as absorbance or luminescent intensity.

493 **Retest date:** A date on which the quality of reference material is evaluated after a
494 specified period of time from the issuance of certificate of analysis.

495 **Selectivity:** The ability of an analytical method to measure and differentiate the analyte
496 in the presence of other components in biological samples.

497 **Specificity:** The ability of an analytical method to detect and differentiate the analyte
498 from similar substances. It is largely dependent on the properties of binding reagent in
499 LBA.

500 **Stability:** The chemical or biological stability of an analyte in a given matrix under
501 specific conditions over given time intervals. Analyte stability is evaluated to ensure that
502 the analyte concentration is not affected as the samples move through each step of the
503 process from collection to final analysis.

504 **Study sample:** A biological specimen that is obtained from a toxicokinetic study or
505 clinical trial for bioanalysis.

506 **Surrogate matrix:** A matrix used as an alternative to a matrix of limited availability
507 (e.g., tissue, cerebrospinal fluid, bile). Surrogate matrix may also be used as an
508 alternative to a matrix that contains endogenous substances that are structurally the same
509 as the analyte.

510 **Total error:** Sum of absolute accuracy and precision.

511 **Upper limit of quantification (ULOQ):** The highest concentration of analyte in a
512 sample at which the analyte can be quantified with reliable accuracy and precision.

513 **Validation:** Demonstration of adequate reproducibility and reliability of an analytical
514 method through various evaluations.

515 **Working solution:** A non-matrix solution prepared by diluting the stock solution in an
516 appropriate solvent. It is mainly added to matrix to prepare calibration standards and QC
517 samples.

518