Administrative Notice
September 13, 2013

To: Prefectural Pharmaceutical Affairs Section

Evaluation and Licensing Division,
Pharmaceutical and Food Safety Bureau,
Ministry of Health, Labour and Welfare

English version of the "Guideline on Bioanalytical Method Validation in Pharmaceutical Development"

We ask you to inform related parties under your administration of this matter as shown in Appendices 1 and 2.

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Appendix 1

Guideline on Bioanalytical Method Validation in Pharmaceutical Development

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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 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 validation of analytical methods applied to measure concentrations of drugs and their metabolites 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 low-molecular-weight drugs (except for endogenous substances), by analytical methods such as liquid chromatography (LC) and gas chromatography (GC) used in combination with mass spectrometry (MS) or with the other detectors.

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 in 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 (purity), and storage conditions should accompany the standard. As a reference standard, it is advisable to obtain a material of known chemical structure from an authenticated source and clarify the expiration date. A certificate of analysis is not necessarily required for an internal standard, but the lack of analytical interference with the analyte should be demonstrated before use as the internal standard.

4. Analytical Method Validation

An analytical method validation should be performed when establishing a bioanalytical method for quantification in every facility.

4.1. Full validation

A full validation should be performed when establishing a new bioanalytical method for quantification of an analyte/analytes. The objective of a full validation is to demonstrate the assay performance of the method, e.g. selectivity, lower limit of quantification (LLOQ), calibration curve, accuracy, precision, matrix effect, carry-over, dilution integrity, and stability. Generally, a full validation should be performed for each species and matrices (mainly plasma, serum, whole blood, or urine) to be analyzed.

A full validation should also be considered when a new analyte, such as a metabolite, is added to an existing, fully validated analytical method. A full validation is also required when implementing an analytical method from a literature.

The matrix used in analytical validation should be as close as possible to the target study samples, including anticoagulants and additives. When an analytical method is to be established for a matrix of limited availability (rare matrix, e.g., tissue, cerebrospinal fluid, bile), a sufficient amount of matrix cannot be obtained from sufficient number of sources (subjects or animals). In such a case, a surrogate matrix may be used to prepare calibration standards and QC samples. However, the use of a surrogate matrix should be rigorously justified in the course of establishing the analytical method.

4.1.1. Selectivity

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

Selectivity is evaluated using blank samples (matrix samples processed without addition of an analyte or internal standard) obtained from at least 6 individual sources. The absence of interference with each analyte and its internal standard should be confirmed. In case of the matrix with limited availability, it may be acceptable to use matrix samples obtained from less than 6 sources.

The evaluation should demonstrate that no response attributable to interfering components is observed in the blank samples or that a response attributable to interfering components is not higher than 20% of the response in the LLOQ for the analyte and also not higher than 5% of the internal standard.

4.1.2. Lower limit of quantification

The LLOQ is the lowest concentration of an analyte at which the analyte can be quantified with reliable accuracy and precision.

The analyte response at the LLOQ should be at least 5 times the response of that in a blank sample. Mean accuracy and precision at the LLOQ should be within ±20% deviation of the nominal (theoretical) concentration and not more than 20%, respectively.

4.1.3. Calibration curve

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

A calibration curve needs to be prepared for each analyte. The 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 a blank sample, a zero sample (blank sample spiked with internal standard), and at least 6 concentration levels of calibration standards, including an LLOQ sample. In general, the simplest model that adequately describes the concentration-response relationship should be used for regression equation and weighting conditions of the calibration curve. A non-linear regression equation may be used. Blank and zero samples should not be included in the determination of the regression equation for the calibration curve. The validation report should include the validated regression equation.

The accuracy of back calculated concentrations of each calibration standard should be within ±20% deviation of the theoretical concentration at the LLOQ, or ±15% deviation at all the other levels. At least 75% of the calibration standards, with a minimum of 6 levels, including the LLOQ and the highest levels, 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. In the validation, QC samples with a minimum of 4 different concentrations (LLOQ and low-, mid-, and high-levels) 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 75% of the upper limit of the calibration curve. Within-run accuracy and precision should be evaluated by at least 5 replicates at each concentration level in a single analytical run. Between-run accuracy and precision should be evaluated by the analysis in at least 3 analytical runs.

The mean accuracy at each concentration level should be within ±15% deviation of the theoretical concentration, except at the LLOQ, where it should be within ±20%. Precision of concentrations determined at each level should not exceed 15%, except at the LLOQ, where it should not exceed 20%.

4.1.5. Matrix effect

Matrix effect is an alteration of the analyte response due to matrix component(s) in the sample. Matrix effect should be assessed when using mass spectrometric methods.

Matrix effect is evaluated by calculating the matrix factor (MF). The MF is determined by comparing the analyte response in the presence of matrix with that in the absence of matrix. MF should be calculated using matrix from at least 6 different sources. The MF may be normalized by its internal standard. The precision of the MF calculated should not exceed 15%.

Matrix effect can also be evaluated by analyzing QC samples, each prepared using matrix from at least 6 different sources. The precision of determined concentrations

should not be greater than 15%.

In case the matrix is of limited availability, it may be acceptable to use matrix obtained from less than 6 sources.

4.1.6. Carry-over

Carry-over is an alteration of a measured concentration due to a leftover analyte in the analytical instrument.

The carry-over should be evaluated by analyzing a blank sample following the highest concentration calibration standard. The response in the blank sample obtained after the highest concentration standard should not be greater than 20% of the analyte response at the LLOQ and and also not greater than 5% of the response of internal standard.

If the criteria cannot be met, the impact of carry-over needs to be examined, and appropriate procedures should be taken to avoid any biases during the analysis of actual study samples.

4.1.7. Dilution integrity

If samples require dilution before analysis, the dilution procedure should be tested to confirm no impact on the measured concentration of the analyte.

Dilution integrity should be evaluated by at least 5 replicates per dilution factor after diluting a sample with blank matrix to bring the analyte concentration within the calibration range. The dilution factors should be selected by considering the dilution method used for study samples. Mean accuracy and precision in the measurements of diluted samples should be within ±15% deviation of the theoretical concentration and not more than 15%, respectively.

If a surrogate matrix is used for sample dilution, the impact on the accuracy and precision should be demonstrated in the same manner.

4.1.8. 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; stability in the processed samples should also be considered. 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 solutions is usually evaluated using solutions near the highest and lowest concentration levels. The evaluation is performed by at least 3 replicates at each concentration level.

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 ±15% deviation of the theoretical concentration, in principle. If the other criteria are more appropriate for the evaluation of 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 subjected to a partial validation are as follows: analytical method transfers between laboratories, changes in analytical instruments, changes in calibration range, changes in sample volume used for analysis, changes in anticoagulant, changes in sample-processing procedures or 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 at both laboratories or by both analytical methods, and the mean accuracy at each concentration level or the assay variability 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 ±20% deviation of the theoretical concentration, considering intra- and inter-laboratories precision. When using a set of study samples, the assay variability should be within ±20% 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 variability) should be separately defined based on scientific judgment according to the type of the analytical methods.

5. Analysis of Study Samples

Study samples are biological specimens that are obtained from toxicokinetic studies and clinical trials Analysis of study samples should be carried out using a fully validated analytical method In the analysis, study samples should be handled under conditions that are validated for adequate stability, and analyzed within a confirmed stability period, along with a blank sample, a zero sample, calibration standards at a minimum of 6 concentration levels, and QC samples.

Validity of the analytical method during study sample analysis should be evaluated in each analytical run by using the calibration curve and QC samples. In studies that serve pharmacokinetic data as a primary endpoint, reproducibility of the analytical method should be confirmed for each representative study per matrix by performing incurred sample reanalysis (ISR: reanalysis of incurred samples in separate analytical runs on different day to determine whether the original analytical results are reproducible).

If carry-over is a concern for the study samples analyzed, the evaluation of validity should also include the item.

5. 1. Calibration curve

A calibration curve is used to determine the concentration of the analyte of interest in study samples. A calibration curve used in study sample analysis should be generated for each analytical run by using the validated analytical method. The same model as in

the bioanalytical method validation should be used for the regression equation and weighting conditions of the calibration curve.

The accuracy of back calculated concentrations of calibration standards at each level should be within ±20% deviation of the theoretical concentration at the LLOQ, or ±15% deviation at all other levels. At least 75% of the calibration standards, with a minimum of 6 levels, should meet the above criteria.

In case the calibration standard at the LLOQ or the highest level did not meet the criteria in study sample analysis, the next lowest/highest-level calibration standard may be used as the LLOQ or the upper limit of the calibration curve. Even though narrowed, the modified calibration range should still cover at least 3 different QC sample levels (low-, mid-, and high-levels).

5. 2. QC samples

QC samples are analyzed to assess the validity of the analytical method used for calibration curve and study sample analysis.

QC samples with a minimum of 3 different concentration levels (low-, mid-, and high-levels) within the calibration range are analyzed in each analytical run. Usually, the low-level is within 3 times the LLOQ, the mid-level is in the midrange of the calibration curve, and the high-level needs to be at least 75% of the upper limit of the calibration curve. The analysis requires 2 QC samples at each QC level or at least 5% of the total number of study samples in the analytical run, whichever is the greater. QC samples should be placed before and after study sample analysis.

The accuracy in the measurements of QC samples should be within ±15% deviation of the theoretical concentrations. At least two-thirds of the QC samples and at least 50% at each concentration level should meet the criteria.

5. 3. Incurred samples reanalysis (ISR)

In bioanalysis, it can happen that the results of analyses of study samples are not reproducible, even when the method validation is successfully conducted and the validity of at each analytical run is confirmed by calibration standards and QC samples. Such failures can be attributed to various factors, including inhomogeneity of study samples, contamination and other operational errors, and interference of biological components unique to the study samples or of unknown metabolites. ISR refers to reanalysis of incurred samples in separate analytical runs on different days to check

whether the original analytical results are reproducible. Confirmation of the reproducibility by ISR improves the reliability of the analytical data. In addition, a failure to demonstrate the reproducibility of the original data in the ISR can trigger a cause investigation and remedial measures for the analytical method.

Usually, ISR is performed for representative studies selected for each matrix in studies that use pharmacokinetic data as the primary endpoint. For instance, ISR should be conducted in the following situations: toxicokinetic studies for each different species; clinical studies representative pharmacokinetic studies for healthy volunteers and patients with renal/hepatic impairment, as well as in bioequivalence studies. For non-clinical studies, ISR may be performed with samples obtained in a independent non-GLP study, if the study design is similar to the relevant toxicokintics study, e.g. sampling conditions.

ISR should be performed with samples from as many subjects or animals as possible and should usually include those of near the maximum blood concentration (Cmax) and the elimination phase. ISR should be performed within a time window that ensures the stability of the analyte. As a guide, approximately 10% of the samples should be reanalyzed in cases where the total number of study samples is less than 1000 and approximately 5% of the number of samples exceeding 1000 samples.

The results of ISR are evaluated using assay variability. Assay variability can be calculated as the difference between the concentration obtained by ISR and that in the original analysis divided by their mean and multiplied by 100. The assay variability should be within ±20% for at least two-thirds of the samples analyzed in ISR. In case the ISR data failed to meet the above criteria, cause investigation should be conducted for the analytical method and necessary measures should be taken by considering the potential impact on study sample analysis.

It should be noted that ISR is performed to monitor assay variability. The original data should never be discarded or replaced with the reanalysis data even if the assay variability exceeds ±20% in a specific sample.

5. 4. Carry-over

Should there be any concern that carry-over may affect the quantification of analyte in study samples, it should be evaluated during the actual study sample analysis using the same procedure described in 4.1.6 to assess the impact on the concentration data in each analytical run.

6. Points to note

6.1. Calibration range

If concentration data obtained during the analysis of study samples are found within a narrow range of the calibration range, it is advisable to redefine the concentration levels of QC samples accordingly.

In case the calibration range is changed, partial validation should be performed. However, it is not necessary to reanalyze the study samples that have been quantified prior to the change (the calibration range, levels or number of QC samples).

6.2. Reanalysis

Possible reasons and procedures for reanalysis, as well as criteria for handling of concentration data should be predefined in the protocol or standard operating procedure (SOP).

Examples of reasons for reanalysis are as follows: calibration curve or QC samples failed to meet the criteria for the validity of analytical run; the obtained concentration was higher than the upper limit of the calibration range; the analyte of interest was detected in pre-dose or placebo samples; improper sample processing or malfunction of equipment; defective chromatogram; and causal investigation on the abnormal value. Reanalysis of study samples for a pharmacokinetic reason should be avoided, whenever possible. In bioequivalence studies, it is not acceptable to reanalyze study samples only because the initial data were pharmacokinetically questionable in order to replace the concentration data. However, reanalysis of specific study samples are acceptable when, for instance, the initial analysis yielded an unexpected or anomalous result that may affect the patient safety in a clinical trial.

In any case, when reanalysis is performed, the analytical report should provide information of the reanalyzed samples; the reason for reanalysis; the data obtained in the initial analysis, if any; the data obtained in the reanalysis; and the final accepted values and the reason and method of selection.

6.3. Chromatogram integration

Procedures for chromatogram integration and re-integration should be predefined in the protocol or SOP.

In case chromatogram re-integration is performed, the reason for re-integration should be recorded and the chromatograms obtained both before and after the re-integration should be kept for future reference.

6.4. System suitability

Analytical instruments used in bioanalysis should be well maintained and properly serviced. In order to ensure optimum performance of the instrument used for bioanalysis, it is advisable to confirm the system suitability prior to each run, in addition to periodical check. However, confirmation of the system suitability is not mandatory in bioanalysis, because the validity of analysis is routinely checked by evaluation of calibration curves and QC samples in each analytical run.

6.5. Recovery

Recovery is a measure of the efficiency at which an analytical method recovers the analyte through the sample-processing step. In order to elucidate the nature of analytical method, it is advisable to evaluate the recovery.

The recovery is determined by comparing the analyte response in a biological sample that is spiked with the analyte and processed, with the response in a biological blank sample that is processed and then spiked with the analyte. It is important to demonstrate the reproducibility, rather than to show a higher recovery rate.

7. Documentation and Archives

In order to ensure adequate reproducibility and reliability of bioanalysis, results obtained in analytical method validations and study sample analyses should be documented in a validation report and a study sample analysis report as described below. The reports should be stored along with relevant records and raw data in an appropriate manner.

All relevant records and raw data should be kept, including those obtained in rejected analytical runs, specifically record of reference materials and blank matrices (receipt/release, use, storage), record of samples (receipt/release, preparation, and storage), record of analyses, record of instrument (calibration and settings), record of deviations, record of communications, and raw data such as analytical data and chromatograms.

Validation report

- Summary of the validation
- Information on the reference standards
- Information on the blank matrices
- Analytical method
- Validated parameters and the acceptance criteria
- Validation results and discussion
- Rejected runs together with the reason for rejection
- Information on reanalysis
- Deviations from the protocol and/or SOP, along with the impact on study results
- Information on reference study, protocol, and literature
- Representative chromatograms

Study sample analysis report

- Summary of the study sample analysis
- Information on the reference standards
- Information on the blank matrices
- Information on receipt and storage of study samples
- Analytical method
- Parameters, acceptance criteria, and results of the validity evaluation
- Results and discussion of study sample analysis
- Rejected runs together with the reason for rejection
- Information on reanalysis
- Deviations from the protocol and/or SOP, along with impact on study results
- Information on reference study, protocol, and literature
- Representative chromatograms, as needed

List of relevant guidelines

1) Regarding "the Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals (ICH M3(R2))" PFSB/ELD Notification No. 0219-4 dated February 19, 2010
2) Regarding the "Note for Guidance on Toxicokinetics: The Assessment of Systemic

Exposure in Toxicity Studies." PAB/ELD Notification No. 443 dated July 2, 1996
3) Regarding the "Guideline on Nonclinical Pharmacokinetics." PNSB/ELD Notification No. 496 dated June 26, 1998
4) "Partial Revision of the Guideline on Bioequivalence Studies for Generic Pharmaceuticals." PFSB/ELD Notification No. 0299-10 dated February 29, 2012
5) Revision of the "Q & As concerning the Guideline on Bioequivalence Studies for Generic Pharmaceuticals." Office Communication dated February 29, 2012
6) "Note on Clinical Pharmacokinetic Studies of Pharmaceuticals." PFSB/ELD Notification No. 796 dated June 1, 2001
7) US FDA: Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services, FDA, Center for Drug Evaluation and Research, Center for Veterinary Medicine(2001)
8) EMA: Guideline on bioanalytical method validation, EMEA/CHMP/EWP/192217/2009, Committee for Medicinal Products for Human Use(2011)

Glossary

Accuracy: The degree of closeness of a concentration determined by the method to the nominal (theoretical) concentration of the analyte. Accuracy is expressed as a percentage relative to the theoretical concentration.

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

Analysis: A series of analytical procedures from sample processing to measurement on an analytical instrument.

Analyte: A specific compound being analyzed. It can be a drug, biomolecule or its derivative, metabolite, and/or degradation product in a sample.

Analytical run: A set of samples comprising calibration standards, QC samples, and study samples. A set of subsequently processed samples, called a batch, is usually analyzed as a single run without interruption in time and by the same analyst with the same reagents under the same conditions.

Assay variability: The degree of difference between the duplicate concentrations determined for a single sample. The difference is expressed as a percentage relative to the mean of the two.
Assay variability (%) = [(Concentration in analysis to be compared - Concentration in reference analysis)/Mean of the two] × 100.

Blank sample: A matrix sample processed without adding an analyte or internal standard.

Calibration curve: The relationship between the theoretical concentration and the response of the analyte. A calibration curve is generated from a blank sample, a zero sample, and at least 6 concentration levels of calibration standards, including an LLOQ sample.

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

Carry-over: An alteration of the measured concentration due to a leftover analyte in the analytical instrument used.

Cross validation: A validation performed when two or more analytical methods are

used within the same study or when comparing analytical methods used in different studies after full or partial validation.

Dilution integrity: Assessment of the sample dilution procedure, when required, to confirm that the procedure does not impact the measured concentration of the analyte.

Full validation: Demonstration of all the validation items i.e., selectivity, lower limit of quantification (LLOQ), calibration curve, accuracy, precision, matrix effects, carry-over, dilution integrity, and stability. Full validation is usually performed when establishing a new analytical method.

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

Incurred sample reanalysis (ISR): Reanalysis of a portion of the incurred samples in separate analytical runs on different days to check whether the original analytical results are reproducible.

Internal standard (IS): A compound added to samples for normalization of the recovery of an analyte during sample processing and the response obtained by the analytical instrument. A structurally similar analogue or a stable isotope-labeled compound is used.

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

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

Matrix effect: An alteration of the analyte response due to matrix component(s) in the sample.

Matrix factor (MF): The ratio of the analyte response in the presence of matrix to the response in the absence of matrix.
MF = Analyte response in the presence of matrix/Analyte response in the absence of matrix.

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

method. It can range from as little as within-run accuracy and precision evaluation to a nearly full validation.

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

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

Processed sample: A sample after processing of a biological specimen, ready for measurement on an analytical instrument.

Quality control (QC) sample: A sample spiked with the analyte of interest to a known concentration used to assess the performance and reliability of an analytical method. In analytical runs, QC samples are analyzed to assess the validity of the analytical method used for calibration curve and study sample analysis.

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

Reanalysis: Repetition of a series of analytical procedures from the processing step on samples that have been analyzed once.

Recovery: The efficiency at which an analytical method recovers the analyte through the sample-processing step.
Recovery (%) = (Response in a biological sample that was spiked with the analyte and processed/Response in a biological blank sample that was processed and then spiked with the analyte) × 100.

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

Response（Response variable）: A response obtained by the detector on an analytical instrument, usually refers to a peak area (or a peak height) obtained from the chromatogram generated by conversion of instrument responses into electric signals.

Selectivity: The ability of an analytical method to measure and differentiate the analyte and the internal standard in the presence of other components in samples. Selectivity is often used interchangeably with specificity, but some point out that these two terms

should be distinguished, as specificity is an ultimate form of selectivity. Based on this idea, specificity is generally the ability to detect a single component, while selectivity is defined as the ability to detect a series of substances which share certain characteristics. In other words, selectivity is the ability to differentiate the analyte and the internal standard from other components, which could also be detected.

Specificity: See the definition of "Selectivity."

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

Stock solution: A non-matrix solution of reference material at the highest concentration prepared in an appropriate solvent.

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

Surrogate matrix: A matrix used as an alternative to a matrix of limited availability (e.g., tissue, cerebrospinal fluid, bile).

System suitability: Confirmation of optimum instrument performance using a reference standard solution of the analyte prior to an analytical run.

Tiered approach: A strategy to initially limit the characterization of analytical method and to gradually expand parameters to be characterized and the extent toward a full validation as the development process proceeds. (see Annex)

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

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

Zero sample: A blank sample spiked with an internal standard.

Annex Application of a tiered approach

Metabolites in human are sometimes unknown at the early stage of clinical trials and the sufficient supply of reference material of the metabolite may be delayed. In such cases, the so-called tiered approach may be applied for analytical method validation for efficient pharmaceutical development.

The tiered approach is a strategy to limit the characterization of an analytical method initially and to gradually expand parameters to be characterized and moving toward a full validation as the development process proceeds. Pharmaceutical research and development could be carried out more efficiently by adopting the tiered approach in the early to mid-stages of the development process, enabling early-stage evaluations and facilitating predictions of future development.

However, even when the tiered approach is used, it is advisable to predefine appropriate criteria for the characterization of analytical method based on scientific judgment in order to improve the reproducibility and reliability of concentration data obtained.

1) Viswanathan, C.T., Bansal, S., Booth, B., DeStefano, A.J., Rose, M.J., Sailstad, J., Shah, V.P., Skelly, J.P., Swann, P.G. and Weiner, R.: *AAPS J.*, 9(1), E30-E42(2007)
2) Timmerman, P., Kall, M.A., Gordon, B., Laakso, S., Freisleben, A. and Hucker, R.: *Bioanalysis*, 2(7), 1185-1194(2010)
3) US FDA: Guidance for Industry, Safety Testing of Drug Metabolites, U.S. Department of Health and Human Services, FDA, Center for Drug Evaluation and Research (2008)

Appendix 2

Questions and Answers (Q&A) for the Guideline on Bioanalytical Method Validation in Pharmaceutical Development

<< Reference standard >>

Q1. How should I use a reference standard when the expiration date has not been established?

A1. When the expiration date is not established, an appropriate quality control for the reference standard by setting a retest date or by using other measures should be employed.

<< Selectivity >>

Q2. Selectivity is one of the parameters to be assessed in an analytical method validation. Is it different from “specificity”?

A2. “Selectivity” is listed as a parameter to be assessed according to the guidelines for analytical method validation. It is defined as the ability of an analytical method to detect the target analyte and its internal standard without having any interference from other components in the samples. “Selectivity” is equivalent to “specificity” which is mentioned in “Text on Validation of Analytical Procedures” (Notification No. 755 of Pharmaceuticals and Cosmetics Division, Pharmaceutical Affairs Bureau, Ministry of Health, Labour and Welfare, dated July 20, 1995). The term “selectivity” is widely used in bioanalytical method validations using chromatography; in addition, “selectivity” has been used in overseas guidances/guidelines. Thus, the term “specificity” in an old dataset can be regarded to an equivalent parameter to “selectivity” mentioned in this guideline.

<<Stability>>

Q3. Can I use an index other than mean accuracy for stability assessment?

A3. In principle, stability of an analyte should be assessed by the mean concentration against its nominal value considering an assay error in the measurement of pre-storage samples. If the other indices are more appropriate for evaluating the stability of a specific analyte in view of

assay precision, indices such as residual ratio could be used for evaluation. When indices such as residual ratio are used, the procedures and acceptance criteria should be predefined in the protocol or the standard operating procedure (SOP) for the evaluation.

Q4. How should I assess stability of an analyte after freeze and thaw cycles?

A4. Quality control (QC) samples stored under the target frozen state are thawed under the same condition as that used for study sample analysis. After the samples are completely thawed, the samples are frozen again under the same condition. The samples should be frozen for at least 12 hours. A series of process from freezing to thawing is defined as 1 cycle, and the QC samples are measured after the same number of freeze-thaw cycles applied to the study samples or more. The accuracy of QC samples should be within ±15% deviation of the theoretical concentration,.

<< Cross validation >>

Q5. What is a specific example of cross validation comparing analytical methods used in different studies?

A5. Cross validation is performed to compare different analytical methods based on different analytical principles (for example, LC-MS/MS and ELISA). In this case, both the validation procedure and the acceptance criteria (i.e., mean accuracy or assay variability) should be separately defined on the basis of scientific justification by considering the nature of the analytical methods.

If analytical methods with the same analytical principle with a minor modification are used in different studies, cross validation may be not performed in most cases, because the validity of the modified analytical method is usually verified by a partial validation.

Q6. Why does this guideline state, “the mean accuracy…at each level should be within ±20% deviation of the theoretical concentration”?

A6. The guideline requires that the mean accuracy of an analytical method at each concentration level should be within ±15% deviation of the theoretical concentration. For cross validation, acceptance criteria is set at 20% considering intra- and inter-laboratory precision.

If study samples are analyzed by different laboratories in the single study, an effort to minimize

inter-laboratory variations is necessary in addition to the analytical method validation. A handling of study samples and reference standards should be defined in the protocol or SOP for the analysis.

<< Incurred samples reanalysis (ISR) >>

Q7. Is ISR required for urine samples?

A7. ISR is mandatory for urine samples as well as for blood samples, if drug concentrations in urine are used as a primary endpoint in bioequivalence studies since no drug is detected in the blood. The need for ISR depends on the significance of urine concentrations.

Q8. How should I perform ISR in toxicokinetic studies?

A8. In a GLP toxicokinetic study, ISR should be performed once per matrix for each animal species. If an analytical method is modified or analysis is performed in a different laboratory, ISR should be performed again.

In addition, ISR can be performed during a bioanalytical method validation using study samples obtained from a non-GLP study such as a dose-finding study performed before a GLP toxicokinetic study. In this case, the study design, including dose and regimen, should be comparable to that of the GLP study.

Q9. How should I perform ISR in clinical trials?

A9. ISR should be performed in representative clinical trials whose pharmacokinetic data as a primary endpoint. To evaluate the validity of an analytical method in an early stage, ISR should be performed as early as possible in the process of pharmaceutical development.

In a clinical trial with a different population of subjects with altered matrix composition, ISR should be performed again. In a bioequivalence study which serves pharmacokinetic data as the primary endpoint, ISR should be performed in the study.

Q10. If study samples obtained from clinical trials are already available at the time of analytical method validation, can I use the samples for ISR?

A10. If you have already obtained study samples from a clinical trial at the time of analytical method validation, you can use the samples for ISR. For example, a metabolite is added to the analyte(s), or reanalysis is performed with an improved analytical method after a failure to meet ISR acceptance criteria. However, an informed consent must be obtained from each subject who provides the study samples. The procedures of ISR and related items should be predefined.

Q11. If overall results meet the ISR acceptance criteria, but the assay variability of a specific sample exceeds the threshold of ±20%, is it required to reanalyze the samples to correct first value?

A11. ISR is intended to confirm the validity of an analytical method using study samples. Therefore, reanalysis of individual study samples is not required to correct the first value, even though the assay variability exceeds the threshold of ±20% when overall result meets the ISR acceptance criteria.

Q12. Where in a report is appropriate to provide ISR results?

A12. When the ISR is performed in the study sample analysis, ISR results should be reported in a study sample analytical report to prove the validity of an analytical method. When the ISR is performed in the analytical method validation, ISR results should be reported in a validation report.

<< Carry-over during study sample analysis >>

Q13. Is it required to repeat assessment of carry-over during study sample analysis even if it is examined in the analytical method validation?

A13. The extent of carry-over may alter depending on the state of the analytical instrument used and the total number of samples analyzed. Thus, carry-over after the analytical method validation should be paid attention. In particular, carry-over should be assessed during study sample analysis, if carry-over cannot be avoided completely in the analytical method validation.

It is not required to report the carry-over in each assay run in a report of study sample analysis.

<< Reanalysis >>

Q14. What issues should be addressed in reanalysis for a pharmacokinetic reason?

A14. Reanalysis of study samples for a pharmacokinetic reason should be avoided, whenever possible, in order to maintain objectivity. If reanalysis due to pharmacokinetic reason is performed, the selection of reanalysis samples should be carefully considered, for example, are included the one before and one after blood sampling points of the questionable sample in the analytical run. In addition, procedures for reanalysis should be predefined, including the number of repeat and the selection of report values, in the protocol or SOP.

In principle, reanalysis of the study samples based on the analytical results obtained is not acceptable in a study using bioanalytical concentrations as a primary endpoint, such as bioequivalence studies. However, this does not restrict reanalysis for investigation and verification which does not replace the concentration data from first results.

<< Others >>

Q15. How should I perform analytical method validation for endogenous substances?

A15. This guideline does not cover the validation of an analytical method for an endogenous substance (e.g., vitamins, amino acids) in biological samples, even though it is administered as drugs; because such validation may involve some issues that are not appropriate for the application of specifications in this guideline. However, it is recommended to perform appropriate validation according to the specifications in this guideline.

It is acceptable to use an appropriate surrogate matrix to measure concentrations of endogenous substances in biological samples. In this case, the validity of the surrogate matrix should be shown in analytical method validation.