Review Article

Methods for the determination of limit of detection and limit of quantitation of the analytical methods

Abstract

The quality of an analytical method developed is always appraised in terms of suitability for its intended purpose, recovery, requirement for standardization, sensitivity, analyte stability, ease of analysis, skill subset required, time and cost in that order. It is highly imperative to establish through a systematic process that the analytical method under question is acceptable for its intended purpose. Limit of detection (LOD) and limit of quantification (LOQ) are two important performance characteristics in method validation. LOD and LOQ are terms used to describe the smallest concentration of an analyte that can be reliably measured by an analytical procedure. There has often been a lack of agreement within the clinical laboratory field as to the terminology best suited to describe this parameter. Likewise, there have been various methods for estimating it. The presented review provides information relating to the calculation of the limit of detection and limit of quantitation. Brief information about differences in various regulatory agencies about these parameters is also presented here.

Key words:
Detection limit, limit of detection, limit of quantitation, quantitation limit, methods for determination of LOD and LOQ

Introduction

Analytical method development and validation procedures are vital in the discovery and development of drugs and pharmaceuticals. Analytical methods are used to aid in the process of drug synthesis, screen potential drug candidates, support formulation studies, monitor the stability of bulk pharmaceuticals and formulated products, and test final products for release. The quality of analytical data is a key factor in the success of a drug and formulation development program. During the post approval commercial production stage of bulk drugs and pharmaceutical products, the official or in-house test methods that have resulted from the analytical method development and validation process cycle become indispensable for reliable monitoring of the integrity, purity, quality, strength and potency of the manufactured products. There is often a need to transfer methodology from one laboratory to another and/or to include it in official compendia. Such exercises include the use of a method by large numbers of people, in various laboratories across the globe and on instruments manufactured by different manufacturers, thereby causing a greater probability of decreased reproducibility and reliability. These problems can be foreseen and avoided by thorough validation of the analytical method.[1]

Limit of detection (LOD) and limit of quantitation (LOQ) parameters are related but have distinct definitions and should not be confused. The intent is to define the smallest concentration of analyte that can be *detected* with no guarantee about the bias or imprecision of the result by an assay, the concentration at which *quantitation* as defined by bias and precision goals is feasible, and finally the

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Table 1: Comparison of different guidelines for "detection limit" parameter of analytical method validation[1]

Guidelines	ICH	US FDA	AOAC	USP	IUPAC
Definition	Lowest amount of analyte in the sample, which can be detected but not necessarily quantitated under stated experimental conditions	Explicitly not described	Lowest content that can be measured with reasonable statistical certainty	Lowest amount of analyte in the sample, which can be detected but not necessarily quantitated under stated experimental conditions.	Smallest amount of conc. of analyte in the sample that can be reliably distinguished from zero.
Method	1. By visual evaluation 2. Based on S/N ratio Applicable to procedure, which exhibits base line noise Low conc. of analyte is compared with blank 3. Based on S.D. of response and slope LOQ = $3.3\sigma/s$ s – Slope of calibration curve σ – S.D. of response; can be obtained by Standard deviation of blank response Residual standard deviation of the regression line Standard deviation of the y-intercept of the regression line $S_{y/x}$, i.e. standard error of estimate	Not described	Based on more than 20 blank readings	For non-instrumental: Analysis of sample with known concentration of analyte and by establishing minimum concentration at which analyte can be reliably detected. For instrumental: Process for non-instrumental can be adopted. Detection limit should be sufficiently low for analysis of samples with known concentration of analyte above and below the required detection limit	Not specified
Expression/ calculation	If based on visual examination or S/N ratio - relevant chromatogram is to be presented If by calculation/extrapolation estimate is validated by analysis of suitable no. of samples known to be near or prepared at detection limit	Not specified	The mean value of the matrix blank readings ($n \geq 20$) plus three standard deviations of the mean, expressed in analyte concentration	Not specified	Not specified
Acceptance criteria	S/N ratio > 2–3; not specified in other cases	Not specified	Not specified	Not specified	Not specified

ICH – International Conference on Harmonisation, US FDA – United states food and drug administration; AOAC – Association of Analytical Communities; USP – United States Pharmacopoeia; IUPAC – International Union of Pure and Applied Chemistry

concentration at which the analyte can be quantitated with a *linear* response.[2] Comparison of regulatory authorities such as United States Pharmacopoeia (USP),[3] Foods and Drugs Administration (FDA),[4] International Union of Pure and Applied Chemistry (IUPAC),[5] International Conference on Harmonisation (ICH)[6] and Association of Analytical Communities (AOAC)[7,8] for limit of detection and limit of quantitation are produced in Tables 1 and 2, respectively.

Detection and Quantitation Limits (LOD and LOQ)

There are several terms that have been used to define LOD and LOQ. In general, the LOD is taken as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified, under the stated conditions of the test. The LOQ is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated conditions of test.[9]

Although reagent package inserts may state that an assay has a dynamic range that extends from zero concentration to some upper limit, typically an assay is simply not capable of accurately measuring analyte concentrations down to zero. Sufficient analyte concentration must be present to produce an analytical signal that can reliably be distinguished from "analytical noise," the signal produced in the absence of analyte.[10]

However, some common methods[11] for the estimation of detection and quantitation limit are

- Visual definition
- Calculation from the signal-to-noise ratio (DL and QL correspond to 3 or 2 and 10 times the noise level, respectively)

Table 2: Comparison of different guidelines for 'quantitation limit' parameter of analytical method validation[1]

Guidelines	ICH	US FDA	AOAC	USP	IUPAC
Definition	Lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy.	The lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy also called LLOQ (Lower limit of quantification).	The limit of quantitation is the lowest amount of analyte in a sample, which can be quantitatively determined with precision and accuracy appropriate to analyte and matrix considered	Lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy	Not defined
Method	1. By visual evaluation 2. Based on S/N ratio Applicable to procedure, which exhibits base line noise. Low conc. of analyte is compared with blank 3. Based on S.D. of response and slope LOQ = $10\sigma/s$ s – Slope of calibration curve σ – S.D. of response; can be obtained by Standard deviation of blank response Residual standard deviation of the regression line Standard deviation of the y-intercept of the regression line $S_{y/x}$, i.e. standard error of estimate	Preparation of standard curve and lowest conc. on the calibration curve should be accepted as LLOQ if it satisfies following condition. Response at LLOQ = 5 × Response by blank Analyte peak should be identifiable discrete and reproducible with precision of 20% and accuracy of 80%–120%	Not specified	1. By visual evaluation 2. Based on S/N ratio Applicable to procedure, which exhibits base line noise. Low concentration of analyte is compared with blank 3. Based on S.D. of response and slope LOQ = $10\sigma/s$ s – Slope of calibration curve σ – S.D. of response; can be obtained by Standard deviation of blank response Residual standard deviation of the regression line Standard deviation of the y-intercept of the regression line $S_{y/x}$, i.e. standard error of estimate	Not recommended; only recommends expressing uncertainty of measurement as function of concentration
Recommendation	Limit should be validated by analysis of suitable no. of samples known to be near or prepared at quantitation limit.	Not specified	Not specified	Not specified	Not specified
Expression/ calculation	Limits of quantitation and method used for determining should be presented. Expressed as analyte concentration	Not specified	Mean value of the matrix blank reading plus 10 standard deviations of the mean, expressed in analyte concentration.	Expressed as analyte concentration (% or ppb)	Not specified
Acceptance criteria	Not specified	Not specified	Not specified	Not specified	Not specified

ICH – International Conference on Harmonisation, US FDA – United states food and drug administration; AOAC – Association of Analytical Communities; USP – United States Pharmacopoeia; IUPAC – International Union of Pure and Applied Chemistry

- Calculation from the standard deviation of the blank
- Calculation from the calibration line at low concentrations

$$DL/QL = \frac{F \times SD}{b}$$

Where

F: Factor of 3.3 and 10 for DL and QL, respectively

SD: Standard deviation of the blank, standard deviation of the ordinate intercept, or residual standard deviation of the linear regression

b: Slope of the regression line

The estimated limits should be verified by analyzing a suitable number of samples containing the analyte at the corresponding concentrations. The DL or QL and the

procedure used for determination, as well as relevant chromatograms, should be reported.

Signal- to-noise

By using the signal-to-noise method, the peak-to-peak noise around the analyte retention time is measured, and subsequently, the concentration of the analyte that would yield a signal equal to certain value of noise to signal ratio is estimated. The noise magnitude can be measured either manually on the chromatogram printout or by auto-integrator of the instrument. A signal-to-noise ratio (S/N) of three is generally accepted for estimating LOD and signal-to-noise ratio of ten is used for estimating LOQ. This method is commonly applied to analytical methods that exhibit baseline noise.[11]

For chromatography a test sample with the analyte at the level at which detection is required or determined is chromatographed over a period of time equivalent to 20 times the peak width at half-height [Figure 1]. The signal-to-noise ratio is calculated from Equation (1).

$$S/D = \frac{2H}{h} \quad (1)$$

where H is the height of the peak, corresponding to the component concerned, in the chromatogram obtained with the prescribed reference solution, and measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to 20 times the width at half-height h is the peak-to-peak background noise in a chromatogram obtained after injection or application of a blank, observed over a distance equal to 20 times the width at half-height of the peak in the chromatogram obtained.

This approach is specified in the European Pharmacopoeia.[5] It is important that the system is free from significant baseline drift and/or shifts during this determination.

Figure 1 shows examples of S/N ratios of 10:1 and 3:1 which approximate the requirements for the QL and DL, respectively. This approach works only for peak height measurements.

Blank determination

It is assumed that they both have the same variance and are normally distributed. As the curves overlap there is a probability that we could conclude that we have detected the analyte when this is in fact due to the blank signal (false positive, α error or type 1 error). Alternatively, we can conclude that the analyte is not detected when it is in fact present (false negative, β error or type 2 error). When addressing the issue about when an analyte has been detected it is always a matter of risk.[11]

The blank determination is applied when the blank analysis gives results with a nonzero standard deviation. LOD is

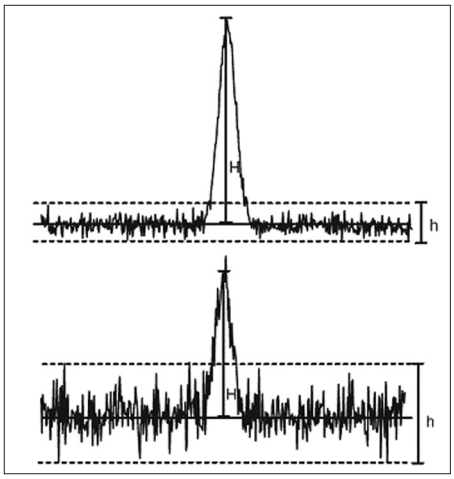

Figure 1: Signal-to-noise examples of 10:1 (top) and 3:1 (bottom), using the method of the EP

expressed as the analyte concentration corresponding to the sample blank value plus three standard deviation and LOQ is the analyte concentration corresponding to the sample blank value plus ten standard deviations as shown in the following equations:

$LOD = X_{b1} + 3S_{b1}$,

$LOQ = X_{b1} + 10S_{b1}$,

where X_{b1} is the mean concentration of the blank and S_{b1} is the standard deviation of the blank. This is a simple and quick method. The weakness is that there is no objective evidence to prove that a low concentration of analyte will indeed produce a signal distinguishable from a blank (zero concentration) sample.[9]

Linear regression

For a linear calibration curve, it is assumed that the instrument response y is linearly related to the standard concentration x for a limited range of concentration.[9] It can be expressed in a model such as

$y = a + bx$.

This model is used to compute the sensitivity b and the LOD and LOQ. Therefore, the LOD and LOQ can be expressed as

$LOD = 3S_a/b$,

$LOQ = 10S_a/b$,

where S_a is the standard deviation of the response and b is the slope of the calibration curve. The standard deviation of the response can be estimated by the standard deviation of either y-residuals, or y-intercepts, of regression lines. This method can be applied in all cases, and it is most applicable when the analysis method does not involve background noise. It uses a range of low values close to zero for calibration curve, and with a more homogeneous distribution will result in a more relevant assessment.

Limit of blank

LoB as the highest apparent analyte concentration expected to be found when replicates of a sample containing no analyte are tested. Note that although the samples tested to define LoB are devoid of analyte, a blank (zero) sample can produce an analytical signal that might otherwise be consistent with a low concentration of analyte. LoB is estimated by measuring replicates of a blank sample and calculating the mean result and the standard deviation (SD).[2]

$LoB = mean_{blank} + 1.645(SD_{blank})$

After calculating this value LOD can be calculated according to $LOD = LOB + 1.645(SD_{low\ concentration\ sample})$.

Precision-based approaches

The quantitation limit can also be obtained from precision studies.[10,11] For this approach, decreasing analyte concentrations are analyzed repeatedly and the relative standard deviation is plotted against the corresponding concentration (precision function). If a predefined limit is exceeded (such as 10% or 20%), the corresponding concentration is established as the quantitation limit However, in practice, due to the high variability of standard deviations the true precision function is much more difficult to draw unless a large number of concentrations is included.

The QL can be specifically calculated[11] using the actual precision of the analytical procedure at this concentration. The calculation is based on the compatibility between analytical variability and specification acceptance limits. QL can be regarded as the maximum true impurity content of the manufactured batch, i.e., as the basic limit

$$QL = AL - \frac{(st_{df,95\%})_{validation}}{\sqrt{n_{assay}}}$$

AL Acceptance limit of the specification for the impurity.

s Precision standard deviation at QL, preferably under intermediate or reproducibility conditions. AL and *s* must have the same unit (e.g., percentage with respect to active, mg, mg/ml, etc.).

n_{assay} Number of repeated, independent determinations in routine analyses, as far as the mean is the reportable result, i.e., is compared with the acceptance limits. If each individual determination is defined as the reportable result, $n=1$ has to be used.

t_{df} Student *t*-factor for the degrees of freedom during determination of the precision, usually at 95% level of statistical confidence.

Conclusion

In this review, the authors have tried to give information to the researchers engaged in establishing analytical profiles of the drug substances or products. Data are adequate and sufficient to meet the laboratory's method requirements. The laboratory must be able to match the performance data as described in the standard and to establish these parameters a manufacturer would test a large number of sample replicates to increase the robustness and the statistical confidence of the estimate. Comparison of all of the validation parameters in different regulatory agencies is summarized by Chandran and Singh[1] and is a better option for curious readers.

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